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Biomedical Materials

TOPICAL REVIEW

Surface engineered nanodiamonds: mechanistic intervention in biomedical applications for diagnosis and treatment of cancer

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Abstract

In terms of biomedical tools, nanodiamonds (ND) are a more recent innovation. Their size typically ranges between 4 to 100 nm. ND are produced via a variety of methods and are known for their physical toughness, durability, and chemical stability. Studies have revealed that surface modifications and functionalization have a significant influence on the optical and electrical properties of the nanomaterial. Consequently, surface functional groups of NDs have applications in a variety of domains, including drug administration, gene delivery, immunotherapy for cancer treatment, and bio-imaging to diagnose cancer. Additionally, their biocompatibility is a critical requisite for their *in vivo* and *in vitro* interventions. This review delves into these aspects and focuses on the recent advances in surface modification strategies of NDs for various biomedical applications surrounding cancer diagnosis and treatment. Furthermore, the prognosis of its clinical translation has also been discussed.

Contents

List of Abbreviations

AD	Alzheimer's disease
ALP	Alkaline phosphatase
ALS	Amyotrophic lateral sclerosis
ABC	ATP-binding cassette
BBB	Blood brain barrier
BMPs	Bone morphogenetic proteins
bFGF	basic-fibroblast growth factor
CpG	Cytosine-phosphate-guanine
CSC	Cancer stem cell
CVD	Chemical vapor deposition
CHO	Cell line
CNS	Central nervous system
DMAPs	Damage-associated molecular patterns
DND	Detonation nanodiamonds
Dox	Doxorubicin
EPR	Enhanced permeability retention
EGRF	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EHMT2	Euchromatic histone-lysine N-methyltransferase 2
FA	Folate
fNDs	Functional NDs
FNDs-Fluorescent nanodiamonds	
FLIM	Fluorescence-lifetime imaging microscopy
FACS	Fuorescence activated cell sorting
GPa	Giga Pasceal (unit of preasure)
GBM	Glioblastoma
H3K9	dimethylation of histone H3 lysine 9
HD	Huntington's disease
HPHT	High-pressure, high-temperature
Kr	Krypton
LSCs	Leukemic stem cells
MDSCs	Myeloid-derived suppressor cells
MLS	Mitochondrial localizing sequence
MMP9	Matrix metalloproteinase 9
MPCVD	Microwave plasma chemical vapour deposition
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
N	Nitrogen
ND	Nanodiamond
Ne	Neon
NCD	Nanocrystalline diamond
FND	Fluorescent nanodiamonds
NKCs	Natural killer cells
NM	Nanometers
NPs	Nanoparticles
NV	Nitrogen-vacancy
N2V	Nitrogen-vacancy-nitrogen
ODN	Oligonucleotides
PD	Parkinson's disease
PEG	Polyethylene glycol
PA	Polyallylamine
PEI	Polyethylenimine
PET	Positron emission tomography
PTT	Phototherapy
PLL	Poly-L-lysine hydrochloride
PDL	Poly (D-lysine)
RES	Reticuloenothelial system
SPECT	Single-photon emission computed tomography
STED	Stimulated emission depletion
TNBC	Triple negative breast cancer
UNCD	Ultra-nanocrystalline diamond

1. Introduction

Nanomaterials are essential in contemporary applications, including targeted therapy, personalized medicine, and nanomedicine, because they integrate therapeutic and diagnostic technologies on a stable theranostic platform [1]. By modifying the chemical, optical, electric, and magnetic properties of materials, nanotechnology enables medical science to advance

treatment for cancer patients, such as reducing the severe side effects of chemotherapy by precisely targeting the delivery of pharmaceuticals, or through improving diagnostic approaches directed towards more efficient cancer diagnosis. Nanomaterials are the fundamental and critical components of nanotechnology. In essence, nanomaterials are materials that are smaller than 100 nm in at least one dimension, i.e. their size is much smaller than that of a microscale [2]. Since the beginning of the nanotechnology era in the 1990s, researchers working on theranostic platforms have concentrated on carbon-based nanomaterials such as fullerenes, carbon nanotubes (CNTs), carbon fibers, graphene, quantum dots, and nanodiamonds (NDs) [3]. Carbon nanoparticles (NPs) are especially well suited for biological applications since the human body and all other existing life on Earth are largely composed of carbon. Since they have an intriguing combination of physical, mechanical, chemical, and optoelectronic characteristics, they enable a variety of applications in catalysis, biology, material science, nanocomposites fillers, and electromechanical equipment.

Carbon NPs with different characteristics pose different hurdles in biological applications. Each carbon-based nanomaterial has its own set of benefits and drawbacks. In the context of biological applications, various carbon-based nano-materials may have certain disadvantages over NDs. Fullerenes are frequently insoluble or only sparingly soluble in water, which may limit their direct usage in aquatic biological contexts [4]. Because of their unique structure, fullerenes, particularly pristine C60, have raised concerns about toxicity in biological systems [5]. Biocompatibility and functionalization of fullerenes for specific biomedical applications can be challenging [6]. While fullerenes have been investigated for drug delivery applications, their imaging characteristics pale compared to NDs. Fullerenes may lack natural imaging capabilities, necessitating the use of additional contrast agents or modifications. CNTs exhibit cytotoxic effects [7], and concerns have been raised about their biocompatibility. The aspect ratio, surface functionalization, and aggregation [8] state of CNTs can influence their interaction with biological systems. The spherical shape and smaller size of ND offer advantages over long and rigid CNTs, especially multi-walled carbon nanotubes (MWCNTs) which induces inflammation and fibrosis in the lungs [9]. CNTs may trigger immune responses, and their immunogenicity can limit their use in certain biomedical applications [10]. Carbon fibers are typically larger and more macroscopic in nature, which can limit their use in applications that require nanoscale interactions, such as drug delivery or imaging at the cellular level [11]. The biocompatibility of carbon fibers may be a concern due to their larger size and potential for mechanical damage to cells and tissues while drug delivery. It can rather be used as a biomaterial for developing scafold for 3D culture in tissue engineering [12]. Carbon fibers' biocompatibility may be a problem because to their bigger size and potential for mechanical injury to cells and tissues during drug delivery. It may instead be employed as a biomaterial in tissue engineering to generate scaffold for 3D culture [12]. The interaction of graphene with cells and tissues might result in oxidative stress and inflammation, which could lead to cellular damage [13]. While graphene has been investigated for imaging and drug delivery applications, significant issues with biocompatibility, dispersion [14], and toxicity may limit its efficacy in these applications. The *in vivo* breakdown of graphene is not entirely known, and there are worries about graphene's long-term destiny in the body [15]. The discovery of quantum dots has enormous effects in biomedical applications. Surface functionalization [16], toxicity, and biocompatibility [17] are the areas of concern. Non-specific cellular absorption of quantum dots may hamper targeted delivery applications [18, 19]. The susceptibility of quantum dots to photobleaching, which results in a gradual decline in fluorescence, limits their long-term imaging capabilities [20]. It is important to note that NDs can provide solutions for the current issues in nanomaterials.

The NDs are among the different carbon family nano allotropes that have drawn the most research interest owing to their inherently unique properties like low toxicity [21], stable fluorescence [22], facile functionalization [23], intrinsic biocompatibility [24] and other basic properties of bulk diamonds. Researchers found ND in areas where nuclear blasts used pyrotechnics with carbon-based triggers [25]. Since then, interest in NDs has disseminated throughout every continent. The discovery of NDs took place over a period of two decades between 1963 and 1982. In addition to detonation, other processes like ion or laser bombardment, electrochemical or ultrasonic synthesis, microwave plasma chemical vapour deposition (MPCVD) techniques, and hydrothermal synthesis are currently used to synthesize ND below 100 nanometers (nm) in size [26]. Additionally, they integrate unique NP characteristics like surface phenomena, and quantum diffusion. Because of these distinctive qualities, NDs have begun to be used in large-scale production just like other carbon-based nanomaterials [27–29]. NDs have extensively researched in the last few years as a possible substance for biological, electronic, and quantum engineering uses due to their low cost, high scalability, ability to be surface functionalized, and besides ensuring high biocompatibility [30].

In the areas of biology and biomedical applications, NDs achieved enormous strides. NDs showed antibacterial activity due to its intrinsic physical properties as well as surface functionalization [31]. NDs

have a variety of physical properties, including size, form, and biocompatibility, making them perfect for formulating individualized nanotherapies like vaccines [32] or drug delivery systems [33, 34]. The beneficial advancements achieved in the field of NDs, focusing on the diagnosis and treatment of infectious illnesses. Furthermore, at a single molecule level, fluorescence imaging is a go-to technique detecting and monitoring inter-cellular interactions and related processes. The ideal features expected in a fluorescent NP include biocompatibility and photostability. Unlike organic dyes employed in biolabeling which undergoes photobleaching or Quantum dots which encounters toxicity, degradation and blinking in spite of having greater brightness and improved photostability, NDs has been observed to present as photostable fluorophores along with overcoming prior mentioned limitations [35]. NDs can serve as excellent imaging tools such as magnetic resonance imaging (MRI), positron emission tomography (PET) and fluorescence imaging, with appropriate surface modifications conjugated with molecular contrast agents externally. Although diamond is considered to have the greatest refractive index compared to other dielectric materials, intracellular medium is estimated twice as much refractive index. Hence Illuminating NDs within cells produces larger backscattered light intensity than the cellular compartments. This was observed when NDs at 55 nm size had a light scattering signal 300 times brighter than similar sized organelles. NDs that are approximately 50 nm in size can be effectively detected by a non-invasive bioimaging technique known as Raman scattering with a sharp peak as demonstrated by studies that involve analyzing the interaction between *E. coli* and lysozyme bound NDs as well as locating growth-hormone receptors using NDs as biomarkers in lung cancer cells [36, 37]. NDs that are used for photoluminescence imaging with negatively charged nitrogen vacancy (NV) color centers is viewed to be a potential alternative that does not undergo photo bleaching or photo blinking but rather exhibits stable brightness. When NV centers undergo excitation specifically with green-yellow light, 700 nm bright red fluorescence which produces a quantum yield that is near 1 in a bulk diamond. These fluorescent properties are hardly affected by ND surface modifications due to the crystal lattice shielding NVs [38, 39].

NDs are powerful enough to interact with and treat a variety of pathogens, including hepatitis B and C viruses [31]. The bulk of research has focused on cancer medicine with the goal of developing tailored therapies for cancer patients using NDs. The hemocompatibility of ND enhances the scope for administering drugs for cancer therapy [40]. Moreover, the ND conjugated drugs achieved a greater height in targeted therapy for cancer treatment [34]. Recent advancements led to the development of ND-based biosensors for metastatic tumor detection [41]. The growth of cancer frequently depends on metastasis, which is also linked to a bad prognosis and drug intolerance. As a result, technological advancements for cancer metastasis biomarker identification can improve both diagnosis and treatment. Matrix metalloproteinase 9 (MMP9) is a metastasis biomarker. Wang *et al* investigated the utility of NDs to develop a stimuli-responsive metastasis recognition complex that employs MMP9 increased expression as a metastasis biomarker. The ND-MMP9 biosensor combination consists of NDs functionalized with peptide substrates that are fluorescently labeled, specific to MMP9, and can detect cancer. Furthermore, among all the types of NPs that have been researched to date for the molecular treatment of neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, Huntington's disease (HD), and amyotrophic lateral sclerosis, NDs have shown the most commitment. Owing to NDs' ability to penetrate the BBB and target particular affected areas of the brain, comprehending the root cause of disease and developing more efficient methods to administer neuromedicine to those specific areas of the brain is achievable [42]. NDs have some flaws that must be surmounted despite being functional substances for drug delivery, cancer research, and treatment. The use of ND as the best carrier for some medications may be restricted by their small surface area, propensity for aggregation, and exposed character. These factors may affect the stability and therapeutic effectiveness of the drug. A well-defined particle's shape, size, and/or surface chemistry have all been understood to be readily modifiable further if needed. Additionally, controlled-release systems show a noteworthy drug loading capacity. Moreover, besides having a pH-responsive controlled-release mechanism that is triggered by certain stimuli for cancer diagnosis and therapeutic application, the material combination should greatly boost photoluminescence. The application of ND can be channeled towards various applications like stem cell therapy [43], provided the NDs biodistribution, and toxicity are taken care of.

In this study, we comprehensively reviewed numerous ND synthesis and characterization methodologies. It is also highlighted how diverse the surface functionalization of ND with different moieties make them suitable for biological applications. The size of ND is crucial in a number of applications since it has been a substantial obstacle in drug administration. ND of smaller sizes, for example, less than 50 nm, would agglomerate and lose usefulness. Thus, focusing on the size of ND, the review discusses several routes related to ND-mediated cancer therapy in which ND is not effluxed or aggregated prior to drug delivery to the target. The use of ND in the treatment of resistant cancer, secondary tumors, and targeted cancer therapies has also been discussed. The biocompatibility aspect of NDs, which has been an important reason for its success in biomedical

applications, is also highlighted. Furthermore, the significance of ND in a variety of medical applications, including the treatment of neurodegenerative disorders, gene therapy, and immunotherapy, has been emphasized.

2. Synthesis and characterization of NDs

2.1. Dynamic synthesis (using explosives)

The detonation technique is a bottom-up approach to ND synthesis used at an industrial scale and was first described in 1963 [44]. Three major methods for synthesizing diamond NPs through the detonation process have been developed in recent years [45]:

i. Direct transformation of carbon precursors (e.g. graphite, carbon black, and coal) to diamond particles in a confined environment by shock waves created by an explosion. High pressure (∼140 GPa) and temperature generated by the shock wave allows for partial conversion of precursors to nanometre-sized diamond grains that further compact to form micron-sized polycrystalline particles [45, 46]. There have been reports of synthetic NDs being produced with dynamic pressure as low as 15 Gpa using other carbon precursors like pyrolytic graphite and CNTs [47, 48]. Here, the NDs synthesized from organic molecules are devoid of metallic contaminants and can be employed in nanomedicine. The high-pressure-high-temperature (HPHT) process has been widely used in the synthesis of NDs which have great toughness, durability, and thermal endurance, making them suitable for use in abrupt cutting, and burnishing tools. The conditions for synthesizing NDs from various carbon sources are severe, with extended development cycles and low production efficacy, making commercial production unfeasible. Thereafter, it is critical to minimize the production temperature and pressure.
ii. Production of ND by detonating a mixture of carbon precursors also uses the principle of shock wave compression. The formation of diamonds in this method occurs within carbon precursors as well as by condensation of carbon atoms present in the explosives. An explosion carried out in air yields a NP size (∼8 nm) smaller compared to an explosion done in an inert atmosphere (<20 nm) [45]. Furthermore, detonated NDs not only exhibit diamond-like firmness, chemical stability, and wear durability, but they also have large surface area that nanomaterials are known for. However, the intense pressure and high temperature created by the explosion have confined the detonation process via wave shock compression to yield ND. Detonation is often performed outside, with a poor yield and revival rate.
iii. Detonation nanodiamonds (DND) are formed by the explosion energy and carbon atoms contained within the explosive molecules used and hence they themselves are the source of precursor carbon [45]. A common explosive used is the combination of TNT (60% wt) & hexogen (40% wt) with negative oxygen balance (i.e. oxygen content lower than the stoichiometric value) such that carbon is present in excess in the system [49]. The explosives are detonated in a closed metallic chamber in a non-oxidizing atmosphere containing gases (e.g. N2, CO2, Ar) or water (ice) for either dry or wet synthesis that further serves as a coolant. This method yields diamond particles of the average size of about 4–5 nm and some other impurities in the soot [45]. The yield of ND depends on the explosive mixtures used, detonation conditions [50] as well as the shape of the explosives [45]. DNDs are inexpensive in cost and have great biocompatibility, therefore they are finding their way into biological applications [51]. However, the high energy consumption and inadequate productivity limit its applicability. Furthermore, NDs produced by detonation often contain graphite, amorphous carbon, and trace metal impurities [51]. As a result, dispersion and purification are essential stages in the generation of NDs in this method.

2.2. Chemical vapor deposition (CVD) technique

CVD technique is another bottom-up approach to ND synthesis and holds an advantage over other methods in that it does not require extensive additional purification of synthesized NDs [52]. Plasma-assisted CVD is generally performed with the seeded non-diamond substrate in either an argon-rich hydrogen-poor environment for Ultra-nanocrystalline diamond (NCD) synthesis (∼2–5 nm) or a hydrogen-lean carbon-rich environment for NCD [53, 54]. There have been reports of ND synthesis by CVD without the use of the substrate. Frenklach *et al* demonstrated for the first time homogeneous nucleation and growth of ND powder directly from the vapor phase of dichloromethane- and trichloroethylene-oxygen mixtures and later acetylene-oxygen mixtures in low-pressure microwave-plasma reactor without the need for the substrate [49]. CVD can potentially create NDs with a predefined density of luminous centers on the substrate that do not require extra purification. This is advantageous for ND's optical and magnetic characteristics. Despite the fact that CVD has drawn a lot of attention as an appealing technique to produce NDs and ND films, this technique's drawbacks must also be considered. For starters, the CVD process often has a sluggish deposition rate, resulting in decreased efficiency. Second, the flammable and poisonous reaction sources and

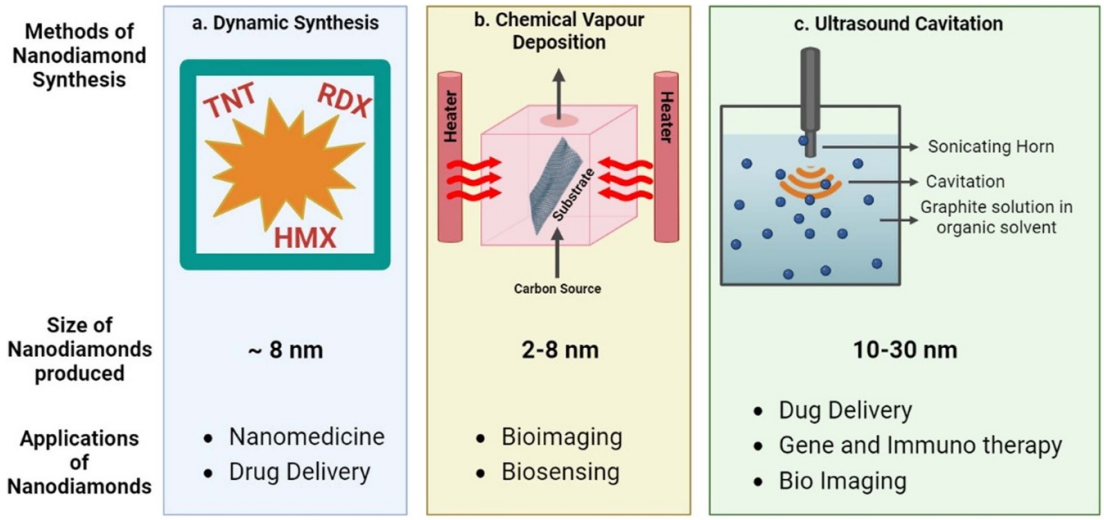

Figure 1. Different techniques of nanodiamond synthesis alongwith their resulting sizes and biomedical applications. (a) Dynamic synthesis (using explosives). (b) Chemical vapor deposition (CVD). (c) Ultrasound cavitation.

reaction byproducts used in CVD necessitate precautions to assure safe manufacturing and avoid contamination. Furthermore, CVD is a relatively expensive method of ND production.

2.3. Ultrasound cavitation

Extreme conditions of high pressure and temperature that are required for the ND synthesis can be achieved by ultrasound cavitation as a result of the rapid collapsing of cavitation bubbles generated in a liquid medium during the process. Galimov confirmed the synthesis of a diamond by exciting cavitation using benzene as both sources of carbon and cavitation media as a result of which particles consisting of aggregates of nanocrystals 10–30 nm in size are produced [50, 55]. Khachatryan *et al* reported mono-crystalline micron-sized diamond particles from graphite powder suspended in organic liquid by using two ultrasonic emitters and also mentioned the mechanism behind formation of the cavitation bubble in their report [56]. The challenge to create a suitable environment for the ND production is dependent on various parameters such as temperature, pressure, amplitude of irradiation, and cavitation fluid. Also, under ultrasonic irradiation, it appears conceivable to make diamond nanowires and nanorods from MWCNTs [57]. Other techniques are properly listed in (table 1) and depicted in (figure 1).

3. Surface engineering in NDs

Because of their abundance of functional groups, NDs are simple for carrying out surface modification. Surface-modified NDs not only have good dispersibility, but they can also interact with a variety of other materials, including pharmaceuticals, fluorescent materials, genetic material, and antibodies, allowing them to meet the requirements of various applications.

3.1. Synthesis and properties

Fluorescent nanodiamonds (FNDs) are a new type of nanostructured carbon-based materials that consist of NDs with fluorescent color centers. Their optical photostability and biocompatibility make them ideal for use in diagnostics, imaging, and therapeutics, all fields that have recently attracted increased interest. The unique optical and magnetic capabilities associated with point defects present in NDs are often put to use in cell labeling and imaging applications. The atomic defects or impurities found in NDs are often quite bright, making them promising candidates for use as fluorescent imaging agents [39]. The majority of the impurities that have been reported to generate optically active defects in ND have been introduced by ion doping, and they include elements like Si, Ge, Ni, Sn, etc [52]. Complexes of impurity atoms attached to inherent structural defects (vacancies and/or carbon interstitials) account for the vast majority of optically active diamond defects. Nitrogen (N) is frequently present in NDs as an impurity, where it replaces a carbon atom. NV centers, which are localized defects consisting of a lattice vacancy neighbouring a substitutional N-atom in the crystalline ND lattice, are created during irradiation and thermal annealing of NDs [66]. Shenderova *et al* found that nitrogen-based flaws produce the most luminous centers, which emit light across the whole visible spectrum and into the near-infrared region (NIR). Vacancies trapped by N-atoms produce different color centers depending on the type of N-atom and its condition within the ND. NV defects, formed by a single N-atom substitution, are responsible for a red/near-infrared fluorescence; nitrogen-vacancy-nitrogen (N2V) defects, formed by

Table 1. Other methods of ND synthesis.

Synthesis method	Particle size	Advantages	Disadvantages	Application	References
HPHT	5–20 nm (also ultrafine ND of <5 nm can be synthesized)	• Uniform structure • minimal lattice defects • simple, two step procedure • highly biocompatible	• long growth cycles • low synthesis efficiency • difficult for large scale production	a. ND and ultra-fine NDs are formed from halogenated hydrocarbons at 8–9 Gpa and >500 °C b. Metal free ND produced by HPHT technique can be used in nanomedicine.	[58–60]
Laser ablation	5–15 nm	• No metallic impurities • Synthesis at ambient conditions • It can be performed in a single step • Can be performed in different liquid media such as water, organic solvents, polymers, acids, salt and alkaline solutions.	• Expensive • ND forms agglomerates in air • Difficult to control particle size and morphology	Laser ablated ND can be used in various fields such as biomedical applications, catalysis, nanophotonics and conductive inks which is further used in flexible printed electronics for screen printing and flexography.	[60,61]
High energy irradiation	2–10 nm	• Efficient production of NV-nanodiamonds • Can be synthesized at room temperature as well as at very high temperature, depending upon the energy radiation	• Production rate of ND is inversely dependent on the amount of energy irradiation by different atoms. Production time may vary depending on the irradiation energy. It may take hours in case of bulk graphite irradiation with subsequent annealing at 800 °C or even femtoseconds using femtosecond laser irradiation of ethanol.	Different irradiation energy is associated with the ND formation, such as, ND is formed in 3 MeV Ne irradiation and 350 MeV Kr irradiation.	[62–66]

a double N-atom substitution, result in a bright green fluorescence [22, 67, 68]. Because of their greater photostability, NV and N2V centers are far more effective than traditional chromophores, even when subjected to continuous high-energy irradiation [38].

3.2. Surface functionalization strategies in NDs

Surfaces of NPs are routinely functionalized with biocompatible moieties to attune them for the application in the biological systems [69]. Although diamonds are generally thought to be inert, they are

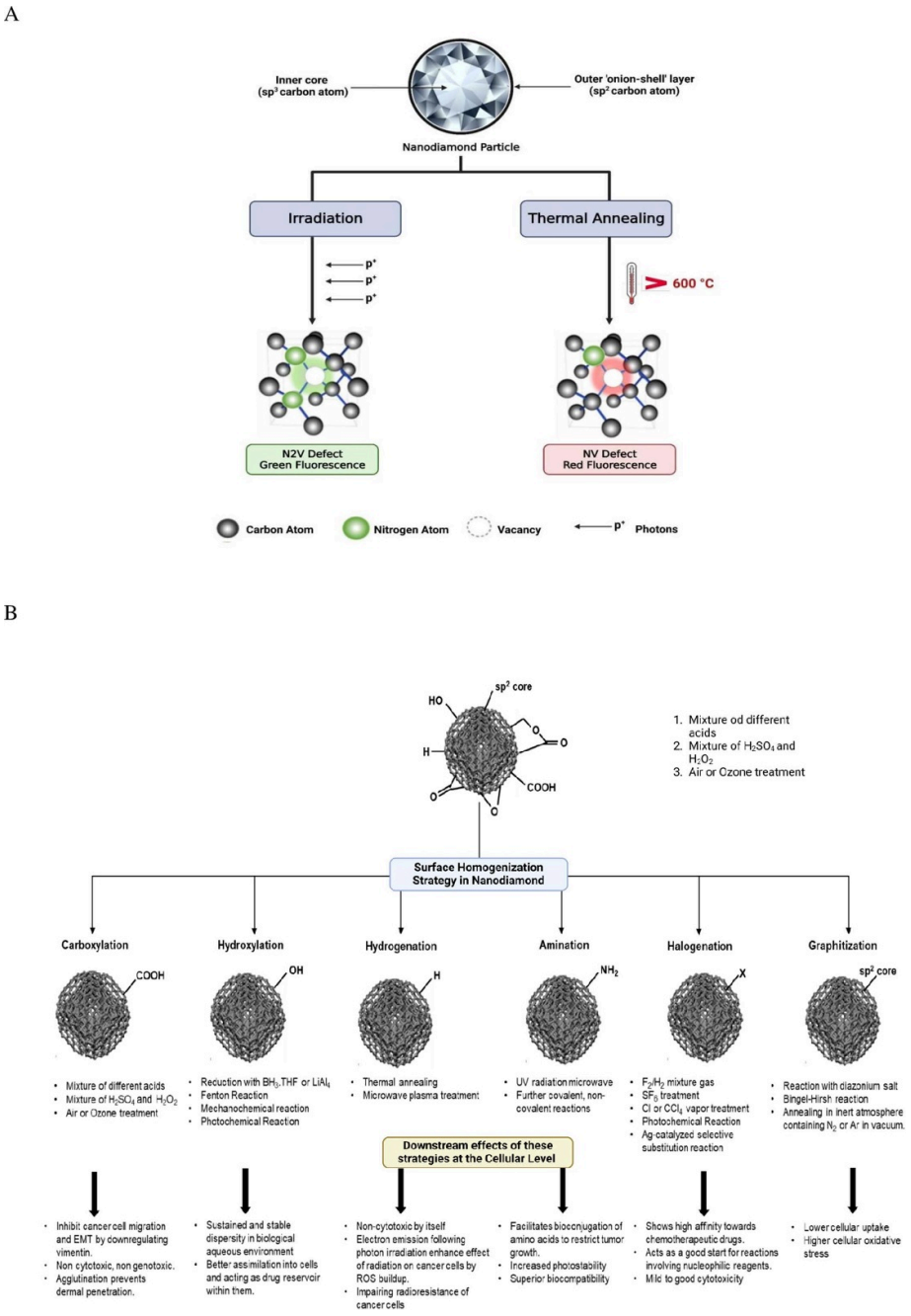

Figure 2. Generation and surface functionalization strategies of of fluorescent nanodiamonds (FNDs). (a) Preparation strategies of FNDs using irradiation and thermal annealing process resulting in different nitrogen defects associated with distinct fluorescence color. Reportedly implemented on nanodiamonds for biomedical applications. (b) Schematic summarizing the protocols of common surface functionalization strategies reportedly implemented on NDs for biomedical applications alongwith the downstream effects of these strategies at the cellular level.

typically known to possess various surface groups which allow for a wide range of surface functionalization strategies [70]. Since color centers are embedded within the ND lattice, surface treatment has almost no effect on their fluorescence properties [39]. Pristine NDs have a broad range of surface moieties, in addition to non-diamond carbon structures and impurities produced during the synthesis process. This has the potential to affect the fluorescence properties of NDs [52]. To enhance purity, regulate subsequent reactions on the ND surfaces, and achieve maximum contribution of NV centers with respect to optical properties of FNDs, these impurities must be removed and surface groups must be homogenized prior to performing further reactions on the surface of NDs. As a result, many studies have been conducted to establish homogenization procedures for NDs, such as carboxylation, hydroxylation, hydrogenation, halogenation, amination, and graphitization [71] (figure 2).

3.2.1. Carboxylation

One of the most popular forms of homogenized ND involves *carboxylation* of ND which takes advantage of rich chemistry of carboxyl (–COOH) groups that can be further subjected to a wide range of treatments to obtain different functional groups []. Carboxylic group can be attached to ND by acid treatment with the mixture of sulfuric acid (H_2SO_4) and Nitric acid (HNO_3) or Hydrochloric acid (HCl) [72]. Alternatively, ND with carboxylic acid is produced during the air ozone purification of ND post synthesis and can be used as starting material for subsequent functionalization [30]. Guo *et al* demonstrated that carboxylated NDs prevented the migration of the tumour cells. It was seen that carboxylation of NDs promoted cell adhesion and restricted the assembly of the cytoskeleton via F-actin staining and AFM-based single cell adhesion experiments [73]. Similar results were also reported in a study by Gao *et al* where the prepared carboxylated ND particles inhibited cell migration in Hela and C6 cell lines when used at concentrations above 100 μg ml^{-1}, and 25 μg ml^{-1} respectively [73]. mRNA studies carried out to decipher the underlying mechanism revealed a concentration dependent decrease in the expression of vimentin, a common regulator of cell migration. It was understood that beyond a critical concentration, the cellular uptake of these NDs increased significantly as a consequence of which cell migration was inhibited, thereby underscoring their utility in cancer therapy. Another experiment was carried out by Liu *et al* where 100 nm carboxylated NDs were distributed to investigate the cell division and differentiation [74]. They observed that the ability of the cell to grow was not altered even after a longer duration of cell culture of both 3T3-L1 embryonic fibroblasts and A549 lung cancer cells. The ND particles were almost equally separated in the two daughter cells during cell division. Moreover, the expression of genes or proteins that control the course of the cell cycle and the differentiation of adipocytes was not affected by ND particles. Collectively, it was concluded that endocytic carboxylated ND particles are non-cytotoxic during cell division and differentiation and can be used to mark and track cancer and stem cells [74].

3.2.2. Hydroxylation

Hydroxylation of ND is among the popular surface homogenization techniques since they also allow for a range of subsequent reactions. Addition of hydroxyl (–OH) group on the surface of ND involves reduction of the oxygenated groups carrying carbonyl (C=O) moieties by wet chemistry method involving borane/THF or using Lithium aluminium hydride ($LiAlH_4$) [75]. $LiAlH_4$ is preferred to borane because it can reduce all kinds of carbonyl functional groups to alcohol (–OH) moieties that borane cannot easily reduce (e.g. esters and lactones) [76]. It should be ensured that all by-products produced as a result of the process are removed. Addition of OH group to the surface by the treatment ND with Fenton reagent has also been reported [77]. Hydroxylated NDs are thermodynamically more stable as previously observed from modeling studies [78]. Studies have shown that hydroxylated NDs have shown potential enhancement of aqueous dispersibility of the drugs in the cancer cells, besides exhibiting better assimilation into cells and acting as drug reservoirs within them ensuring sustained release [79]. Paclitaxel, which is poorly soluble in aqueous medium, showed increased hydrophilicity when conjugated with a hydroxylated ND. Lim *et al* demonstrated that ND-OH/PTX exhibited a higher retention and a sustained release of PTX by about 97.32% at 70 h while bare crystalline PTX only showed 47.33% of drug release capacity. Cell lines like Hela, MCF-9 and A549 exhibited low cell viability due to the enhanced sustained release of PTX from the ND-OH complex and stable dispersity in physiological aqueous environments. This ND-OH/PTX accumulates into the cells and induces initial necrosis in the cancer cells [80].

3.2.3. Hydrogenation

The most efficient approach to produce ND terminated with hydrogen is the *hydrogenation* within the plasma reactor. The use of atomic hydrogen produced by MPCVD allows high control of C–H surface terminations. This leads to the increase in the hydrophobicity of ND because of complete reduction of oxygenated functional groups (C=O, OH and C–O–H) [81]. Regardless of its simplicity and potential utility, this approach raises concerns about the process conditions which restrict the applicability of this procedure as a surface functionalization technique. Grall *et al* demonstrated that the negative electron affinity of hydrogenated nanodiamonds (H-NDs) imparts a high degree of reactivity with oxygen species as well as a positive charge in aqueous solutions. It permits H-NDs to emit electrons after being exposed to photons, which could amplify the effects of radiation on cancer cells [82]. The potentialization of cytotoxicity after irradiation and co-exposure of H-NDs was investigated using radioresistant human cancer cell lines. More intracellular reactive oxygen species (ROS) are produced as a result of the co-exposure (H-NDs and radiation) than from either therapy alone. H-NDs in an aqueous environment are anticipated, based on their properties, to adsorb a significant number of oxygen-related species [83], potentially acting as a source of ROS when triggered by electron photoemission via ionising radiation. Thus, it was concluded that hydrogenation of NDs increased the potential efficacy of radiation in radio-resistant cancer cells, thereby leading to cancer cell death.

3.2.4. Amination

Amination of the ND surface directly confers a wide array of applications allowing the binding of collection of functional molecules like bioactive compounds or polymer building blocks by different mechanisms. The reported method to graft amino group ($-NH_2$) on the surface of ND involves the treatment of chlorinated diamond with ammonia gas at high temperature [76, 84]. Other methods for amination of ND like photochemical method [85], and treatment with ammonia plasma [86] have also been reported. Another group reported grafting of groups carrying primary amino groups using Diels–Alder reaction [87]. Ryu *et al* fabricated a folic acid conjugated ND (FA-ND) complexes for selective photothermal tumour therapy [88]. Ethylenediamine was used to aminate the ND complex with surface carboxyl groups, and carbodiimide chemistry was then used to conjugate those nanoclusters with FA. The higher uptake of FA-ND nanoclusters by folate receptor-positive KB cells over folate receptor-negative WI-38 cells suggests that FA-ND nanoclusters are only effective against tumour cells that overexpress folate receptors. When exposed to a NIR laser, FA-ND nanoclusters efficiently and selectively destroy KB cells, according to cell viability studies. Additionally, photos taken with a fluorescence microscope show that only KB cells treated with FA-ND nanoclusters are ablated in a spot using a NIR laser with a diameter of 200 m. Similarly, for the treatment of cervical cancer, Alwani *et al* created ND-gene complexes known as diamoplexes. Lysine amino acid was covalently conjugated to carboxylated NDs surface produced by reoxidation in strong oxidizing acids throughout the synthetic process. Zeta potential and particle size results indicated minimal sedimentation and good stability. According to the results, functionalizing NDs with lysine maintained their long-term stability and made it possible for them to interact with physiological systems [89].

3.2.5. Halogenation

Halogenation of NDs is a convenient method of surface activation via the creation of electrophilic centers capable of further reaction with multiple nucleophilic reagents. Fluorine can be grafted onto the surface of diamond by reaction with F_2/H_2 mixture at an elevated temperature [90], or by applying atmospheric pressure plasma with CF_4. Chlorinated ND can be obtained by thermal treatment of ND with Cl_2 or CCl_4 [4] or UV irradiation of chlorine gas with hydrogenated ND [91]. Halogenation such as chlorination or fluorination of NDs shows high photostability thus making them ideal for cell tracking, imaging and electron microscopy [22, 91, 92]. Chlorination might reduce the stability of the NDs when present in air due to high reactivity. However, in alkaline medium it exhebits equal stability as fluorination [93]. Fluorination increases the stability of the NDs by replacing the C–C covalent bonds of the diamond lattice by fluorine atoms [94]. These surface modified NDs can be used for tumor cell detection. Additionally, it was demonstrated that the halogenated NDs show higher affinity towards the therapeutic drugs used for cancer treatments [95]. Halogenated NDs offer a good starting point for further reactions involving nucleophilic reagents. The shallow negatively charged NV centre is stabilized by fluorinating the ND surface [96].

3.2.6. Graphitization

Annealing in the inert atmosphere containing N_2 or Ar or in vacuum completely removes the functional groups and induces *graphitization* of surface diamond leading to formation of onion-like shells (with carbon atoms in sp^2 state) around the ND core (having carbon atoms in sp^3 state) [97, 98]. The transformation can also be achieved by high energy electron irradiation [99, 100]. As previously mentioned, synthesized pristine ND contains non-diamond graphitic carbon impurities [101, 102] but their structure and amount cannot be controlled during the fabrication process [103]. So, such impurities need to be removed and again promote graphitization even though sp^2 terminated ND surface is needed [76]. For a better understanding of surface functionalization strategies of NDs, the authors suggest the readers to go through a recent review by Jung and Neuman [104]. Graphitized NDs are suitable as nanoplatforms for intracellular transport due to their distinctive physicochemical characteristics. Their small size makes them easy to transport through the circulatory system, and their large surface area and with the various decoration techniques currently accessible, they can readily have their surface changed and functionalized with fluorophores or numerous ligands, enabling the targeted delivery of medicinal medicines [105]. Thus, to conclude, the graphitized NDs can be used to target cancer cells because of their potential ability to show a sustained release of the therapeutic drugs, low cytotoxicity, and minimal inflammatory responses [106] (table 2).

4. Mechanism behind ND mediated anti-cancer therapy

NDs make for ideal drug carriers, because of their biocompatibility, greatly reduced size and large surface area for specific interactions [110]. Off-target cytotoxicity, drug resistance, and decreased effectiveness from decreased retention and poor circulation are the main challenges in cancer treatment. The employment of NDs has made it possible to get around any obstacles. Because of their prolonged cellular retention, nanocarriers can selectively target cancer cells without overwhelming the body's immune system [111]. In addition to their usefulness *in vivo*, NDs, and particularly detonation NDs, are

Table 2. Advantages and disadvantages of different surface modification.

Sl. No.	Surface modifications	Advantages	Disadvantages	Applications	References
1	Carboxylation	• Non cytotoxic • Restricts cancer cell division and differentiation • Improved permeability • Biocompatible • Shows antioxidant properties • Regulates cancer cell migration	• Presence of high no. of impurities and lattice defects • Cannot keep fluorescent defects stable • Cannot be used for tracking, marking and sensing techniques.	• Facilitates drug delivery • Increased photostability • *In-vivo* imaging	[63, 107]
2	Hydroxylation	• Increases hydrophilicity of chemotherapeutic drugs. • Helps in sustained release of drug • Increases drug retention capacity.	• Presence of impurities • Less stability	• Drug delivery	[80]
3	Hydrogenation	• Imparts high degree of reactivity with oxygen species. • Could amplify the effect of radiation on cancer cells.	• Huge amount of ROS is produced. • Increases hydrophobicity which reduces the drug solubility and release capability	• Radiation therapy for cancer treatment • Electron microscopy	[82]
4	Amination	• Increased photostability • Superior biocompatibility • Facilitates bioconjugation of amino acids to restrict tumor growth.	• Leads to aggregation and clustering of NDs. • Causes inhomogeneous surface reactivity • Possesses complex structure	• Drug delivery • Tumor cell suppression • Gene delivery system	[88, 108]
5	Halogenation	• High photostability • Shows high affinity towards chemotherapeutic drugs • Acts as a good start for reactions involving nucleophilic reagents.	• High consumption of energy • Low productivity • Chlorinated NDs might affect stability in air due to highly reactive property.	• Cell tracking and marking. • High quality imaging of cancer cells • Electron microscopy • Drug delivery	[91, 93]
6	Graphitization	• Provides additional carriers for conductivity • Provides high mobility at high temperatures • Can be operated within a huge range of temperature without losing its properties and performance.	• Structure cannot be controlled during fabrication • High energy consumption • Difficult to eradicate all the impurities.	• Facilitates intracellular transport • Targeted drug delivery	[105, 109]

very amenable to chemical modification via bonding molecules called linker molecules and maintain their stability for months. The large surface area and biocompatibility of cells and whole organisms offer more advantages [110]. Chemotherapy's non-specific cytotoxicity and significant adverse effects can be mitigated with greater treatment efficacy. It has the potential to reduce resistance, shorten treatment times, and reduce doses. Since its uniqueness is still growing, there is room to improve existing therapies and investigate its mechanisms in greater depth.

4.1. Cellular uptake pathways employed for anti-cancer drug action

Owing to their size, NDs are promising drug carriers. The size is also easily modifiable to influence the circulation and accumulation of the particles. As a rule of thumb for NPs, it is considered that particles less than 100 nm can enter the cell, smaller than 40 nm can enter the nucleus, whiles smaller than 35 nm can cross the BBB [112]. Very small particles, like 5 nm in size, are cleared quickly by the kidneys and do not have enough time to show the anti-cancer effect. While large NPs larger than 50 nm will not be cleared by the kidneys, they can be trapped by the reticuloendothelial system (RES) [111]. The accumulation in RES is largely harmless at the organ level. In the exposure time period, if the NDs accumulate in the tumour site, they can act on the cancer. Accumulation is mainly caused by enhanced permeability retention (EPR), a passive movement enhanced by poor vasculature in the tumor [113]. Consequently, various cellular uptake pathways exist that are associated with the internalization of NDs with varying sizes and surface homogenities [114] (figure 3).

Some cell types may phagocytoze NDs, but others may simply allow them to diffuse directly over the cell membrane. The phagocytosis of NDs by macrophages has been reported earlier using phagocytosis inhibitors and through TEM imaging by Niora *et al* [115]. Moreover, variations in ND uptake by THP-1 cells at early exposure times as observed in their study suggest that phagocytic capacity varies. The cell fraction with substantially increased ND absorption is linked to a macrophage phenotype derived from spontaneous monocyte differentiation. These findings show that differences in ND uptake allow for differentiation of cell subtypes based on phagocytic capacity. Overall, NDs successfully mark monocytes and macrophages and have been identified as attractive candidates for monitoring biological processes involving cell differentiation.

It is noted that positively charged NPs are quickly internalized by the negatively charged cell membrane via endocytosis [112]. Progress in surface coating and manipulation has allowed shielding the NDs from macrophage uptake and stabilizing the structure [116]. For example, polyethylene glycol (PEG) coating increases the half-life of the system by reducing RES uptake [117]. Surface specific-targeting antigens can also draw the NDs to the site of tumour via active movement and promote aggregation, increasing anti-cancer action [118, 119]. Another approach to specifically target epidermal growth factor receptor (EGFR), NDs, was used to co-deliver paclitaxel and cetuximab. This combination enhanced cellular uptake in EGFR-expressed colorectal cancer cell line, HCT116 and inhibited microtubule formation and mitotic cycle, leading to apoptosis [120]. NDs loaded with cisplatin, released the drug at a much higher rate at pH 6, than pH 7.4. pH-responsive drug release makes sure that the drug is not released in the bloodstream, but in the acidic tumour microenvironment, thus reducing systemic side effects. Such controlled release makes it possible to explore more pH-dependent surface modifications [121].

Lack of internalization of large NDs (120 μm) in mammalian cells [122] is a disadvantage of these nanocarriers, especially seen in HT-29 colon cancer cell line [123]. Sigaeva *et al* eliminated this problem by studying the physical and spatial delivery of fluorescent NDs. It showed that internalization was higher when treated from the basolateral (bottom) than apical (on top). Further, if the cell clusters are treated with trypsin-EDTA, drastic internalization is observed. The distribution pattern and co-localization was not affected, nor had adverse toxicity [123]. A simple trypsin treatment cleaved the cells, increasing the surface area and perhaps easier penetration. Similarly, to increase biocompatibility and cellular uptake in HT-29, camptothecin was encapsulated in alkyl-amine modified NDs using sonication. It showed biocompatibility on cell lines, i.e. *in vivo* and xenografts in mice, as well as high killing capacity of carcinomas, confirmed by the reduced tumour sizes [124].

Another functionalization of NDs, with PEG was carried out by Madasetty *et al* wherein PEG NDs loaded with doxorubicin were found to be more effective on pancreatic ductal adenocarcinoma in *in vivo* orthotopic xenograft mouse models and *in vitro* 3D models than free Doxorubicin [125]. They were found to be compatible with macrophages and no toxicity was observed. Findings were comparable in 3D models and *in vivo* than 2D models, which underlines the importance of using relevant pre-clinical models for drug screening. A natural yet innovative approach was chosen by Zheng *et al* to improve uptake and stabilize the NDs in biological colloids. They used recombinant protein to guide the entry of these NDs into the cells [116]. The K-12 domain provided electrostatic stability while the C-4 tetramer provided hydrophilicity. It binds to the NDs with weak hydrogen bonds. MTT assays confirmed that this combination did not affect the cells

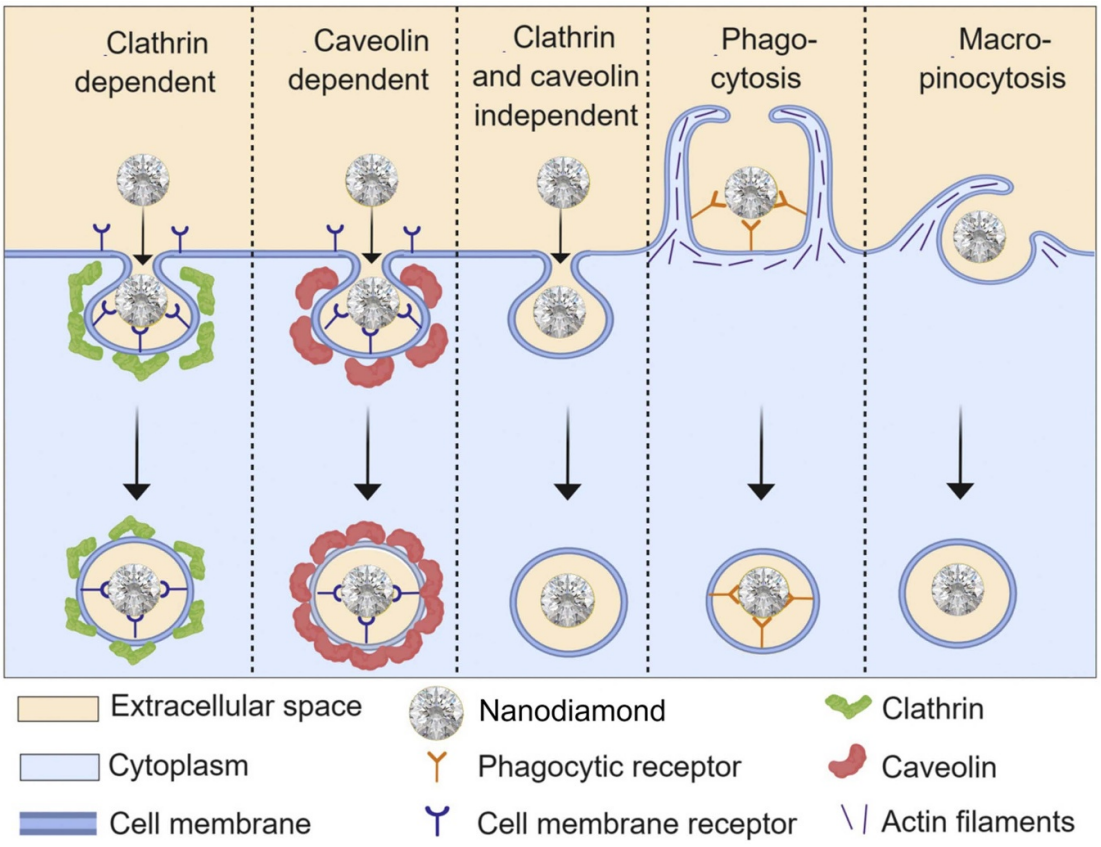

Figure 3. Schematic overview of nanodiamond uptake pathways via endocytosis. There are several avenues for ND entry into cells via endocytosis mechanisms such as clathrin-dependent; caveolin-dependent; clathrin- and caveolin-independent; phagocytosis; and, macropinocytosis. These absorption mechanisms for NDs are mechanistically unique and biomolecularly highly controlled. The route by which the ND enter cells is critical because it dictates intracellular nanoparticle movement, as well as the accompanying biological reaction and therapeutic efficacy. Reprinted from [114], Copyright (2019), with permission from Elsevier.

and were harmless. It also showed improved uptake in HeLa cells and HT29 cells. Such advances also open the pandora of developing new peptides, which can have specific ligands or intracellular, intra-nuclear targets.

Like cytotoxicity, the surface decoration of NDs decides the type of intake pathway they will undergo. Intake of NDs of size 10 nm was studied by Liu *et al* [74]. They concluded that NDs can be taken into the cell (MCF7, 3T3-L1, RKO, HCT116, and lung adenocarcinoma) through macropinocytosis and clathrin-mediated endocytosis. Endocytic vesicles of 0.2 μm to 1 μm can mediate the intake by extension, called filopodia from the cell-surface ruffling. Clathrin, a triskelion structure, also plays an important role in engulfing the 100 nm NDs. These NDs also formed clusters inside the cytoplasm, becoming undegradable and longer acting inside the cell. It was also observed that NDs did not hamper the regular functioning of the cell, like spindle formation for mitosis or segregation. This further strengthens the non-toxicity of NDs. Doxorubicin drug delivery in human liver cancer cells (HepG2) was studied by Wang *et al* in 2013. PEGylated NDs were formed by conjugating carboxylated NDs with hydroxyl-polyethylene glycol (PEG-4000). Cellular uptake was confirmed to be a temperature, energy and clathrin-dependent pathway by flow cytometric analysis. The experiments were conducted in three different conditions: (i) 4 °C-for temperature study, (ii) pre-treated with sodium azide-blocks cellular ATP production and (iii) pre-treated with sucrose-inhibitor of clathrin coated vesicles. The three studies showed reduced uptake of PEGylated NDs loaded with doxorubicin by 85%, 60% and 70% respectively. Therefore, confirming that PEGylated NDs are internalized by clathrin-mediated, energy dependent pathway [126]. Tumour cells overexpress folate receptors that make them targets by folate ligands. PEGylated NDs were conjugated with folate and loaded with doxorubicin [127]. The uptake was mediated by a clathrin dependent pathway and also correlated with the level of folate receptor expression in HeLA and HepG2 cells. Dox was released in a slow and sustainable manner and was also found in the nucleolus. Similar results were obtained in a targeting therapeutic using transferrin by Li and Zhou [128]. The conclusions are however debatable as there are articles which mention that folate receptors intake folate/FA by caveolin-dependent endocytosis [129]. Caveolae are inward

forming invaginations with high lipid content and regulated by cholesterols [130].

Another study involved harsh treatment of the raw detonated NDs with fenton [77]. It helps in removing the soot particles and incorporates –OH groups, which will help in covalent bonding of thionine to the ND. It increased water solubility with the hydroxyl groups and for the first time was observed to enter nuclei of HeLa cells. This method can hence be exploited for gene delivery into the nucleus. Such a gene delivery was tried by attaching two cationic polymers, polyallylamine (PA) and polyethylenimine (PEI) to NDs which will carry the siRNA in Ewing sarcoma cells. Selective inhibition of the clathrin mediated pathway by chlorpromazine severely hindered uptake of ND-PA and to a lesser extent of ND-PEI. While blocking caveolae with filipin, did not show any change in the uptake. Inhibition of macropinocytosis by treatment with amiloride (Na^+/H^+ ATPase exchanger inhibitor), showed partial inhibition of ND-PEI but not of ND-PA. This shows that ND-PA is largely Clathrin dependent and ND-PEI is clathrin and macropinocytosis dependent for internalization. Similar selective inhibitions followed by gene expression studies of siRNA on target mRNA revealed that only NDs intaken by macropinocytosis were showing the inhibitory action [131]. As discussed earlier, the study by Liu *et al* also showed a possibility of macropinocytosis. The images procured by bio-atomic force microscope allowed them to observe the ruffling of cell surface to form filopodia and extend it to come in contact with the ND. Once ingested into the cell, they formed aggregates of NDs vesicles in the cytoplasm. Fluoro tags also showed the presence of clathrin mediated entry into cell [74]. In most of the examples discussed the pathway adopted is clathrin-mediated or macropinocytosis. There are reports of caveolae-mediated pathways for NPs, which are not robust pathways for NDs.

4.2. NDs in treating resistant cancer

NDs have also found use in treating resistant cancer cells through sophisticated mechanisms governed by their inherent characteristics (figure 4). Resistance in cancer cells is caused by various reasons like, drug inactivation, mutation in target receptor, lack of retention in target cells, epithelial-mesenchymal transition and drug efflux from the cell [111, 132]. Chemoresistance due to efflux of drugs is mainly attributed to the ATP-binding cassette (ABC) protein transporters. Over expression of ABC in cancer stem cells (CSCs) leads to poor drug action and disease progression [133]. Some method examples by which ND have been able to treat resistant cancer will be discussed in this segment. Truncated octahedral structure of ND impairs drug efflux from the cancer cells and presents multiple faces for drug binding and easy dispersion in pharmaceutical vehicles [134]. This results in accumulation of drug inside the cancer cells, thus allowing longer action [110]. The intake pathway was majorly clathrin-dependent. Drug retention analysis by Wang *et al* showing that epirubicin loaded onto ND, were retained in the tumour cells, and avoided the ABC transporter. A possible reason for avoiding the ABC receptors could be the revised size and shaped of ND-drug complex. It also effectively targeted CSCs and prevented secondary tumours from forming in *in vivo* models [135]. To avoid the efflux of NDs, Chan *et al* formed a dual ligand ND; mitochondrial localization sequence (MLS) peptide and folate (FA) loaded with doxorubicin. The study carried out on MCF-7 and HeLa cell lines pointed out that FA functionalization allowed to target the tumour cells (with over-expressed folate receptors) and the MLS peptide, drove the ND to the mitochondria of the cell. They showed enhanced uptake as well as cytotoxicity and cell death in the Dox-resistant MCF-7 and HeLa cell lines [136]. Conjugation of a secondary plant metabolite, citropten with NDs has shown to arrest cell cycle. It also altered mRNA levels of cytoskeletal elements, like β-actin deposition and induced morphological changes in B16F10, skin cancer cell line [137]. A similar genetic change was observed in NDs conjugated with PEG and Doxorubicin loaded. ABCG2 is an important receptor for expunging drugs from the tumour and hence imparting drug resistance in cancers. In the mentioned study, by Madasetty *et al* it was observed that ABCG2 had decreased expression as well as decrease in a cancer pro-survival protein called Akt in *in vivo* NSG mice [125]. The decrease in ABCG2 receptors implies that drugs were able to stay in the target cells for longer duration and give better response. *In vitro* studies on PANC-1 and 6741 Cell lines showed an obvious increase in cytotoxicity when conjugated with NDs than free doxorubicin. Such studies open a new topic, where conjugation with ND has allowed change in genetic expression of cancer cells. One inhibits the cell cycle while the latter reducing expression of receptor and proteins that help in cancer progression. Another chemoresistance story in the poorly prognostic and aggressive triple negative breast cancer (TNBC) was studied by Yuan *et al* recently. They used NDs conjugated with polyglycerol and loaded with doxorubicin. This ND-dox, did not upregulate the key chemoresistance mediators in 4T1 cells, i.e. P-gp and IL-6. It also reduced production of myeloid-derived suppressor cells (MDSCs), which suppress the anti-tumour response of the body. This study of tumour microenvironment and systemic immune response to ND-dox, shows that the NDs may have a facet that can be used to amalgamate immunotherapy and chemotherapy [138].

Other than chemotherapy, NDs have also found place in phototherapy (PTT) of glioblastoma and cancer cells. In the past decades carbon allotropes have gained momentum, due to their ability to conjugate with multiple and diverse molecules, enable

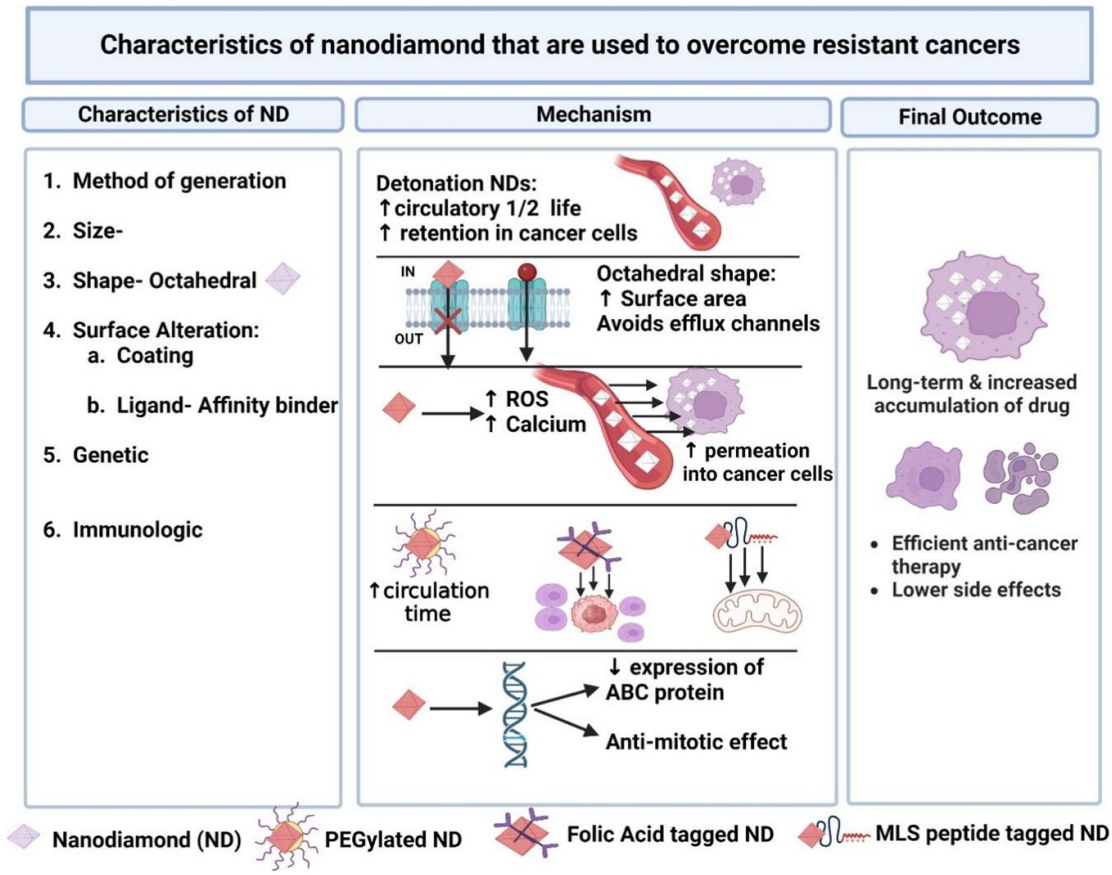

Figure 4. Schematic depiction of the characteristics and mechanisms of nanodiamonds that are exploited to overcome resistant cancer.

bio-imaging, therapeutics and high surface area, which exacerbates the photon-to-thermal energy conversion [139]. PTT involves use of a NIR to ablate cancer cells, using photo-absorbers, called photothermal agents. Since NDs have low toxicity and versatility, it can be combined with PTT. In 2013 a group of scientists studied the viability of J hepatoma cell lines using FND conjugated with Au/Ag NPs, loaded with Transferrin biorecognition [140]. It showed highest biocompatibility and PTT showed it destroyed malignant J5 cells, with photo-translating hyperthermia. Transferrin loaded nano-clusters showed higher uptake [140]. Selectivity can also be achieved by conjugating FA with NDs, forming FA-NDs. FA is positively expressed in carcinoma cells of the mouth, like KB cells. FA-NDs were taken up by KB cells selectively and destroyed by PTT [88]. NDs deliver polydopamine and indocyanine green to specific cancer cells, and will specifically destroy a tumour [141]. A study Yu *et al* involved the creation dual functioning ND, by fluoro-tagging with ICG and loading paclitaxel drug to the ND-supraparticle, forming, PTX-ICG-ND-SP [142].

The approach of using the conversion of a form of energy in phototherapy to treat cancer is easily quantifiable (live tracking labelling), targeted, and least invasive is also gaining momentum, by combining fluorescence and drug delivery. As gene/protein targeting technologies emerge, this method can be used to destroy cells with altered expression profile and possibly in diseases like Alzheimer's and Parkinson's, where there is build-up of $A\beta$ and Tau protein respectively Their abilities also make them NDs and PTT make them a potent tool for diagnostics too. An important hook for the success of PTT, is to control the laser intensity, depending on the cells, photosensitizer and labelling moiety.

5. Biocompatibility of NDs *in vitro* and *in vivo*

As this medium of nanocarriers gains popularity for therapeutics, safety concerns like cytotoxicity and long term implication on organ systems will need to be studied. A study conducted by Schrand *et al* tested the cytotoxicity of NDs of sizes 2 nm to 10 nm [15]. NDs, with modified chemical entities like COOH, –COONa etc, were found biocompatible with various cell types like, macrophages, keratinocytes etc. They also showed that cells can grow on ND-coated substrates like glass cover slips, without any physical changes. Vaijayanthimala *et al* proposed and checked ND with poly-L-lysine hydrochloride (PLL) surface

modification and showed that pristine FND did not have any cytotoxic effects [24]. PLL labelling is often used on stem cells. PLL-ND showed increased cellular uptake in HeLa cells. Biocompatibility in such models ensures unbiased results in future studies, with the cell lines. They both also showed similar uptake mechanisms, using clathrin-mediated, claveole-independent and energy dependent pathways. Cytotoxicity of peptide-grafted carboxylic NDs on CHO cell line was studied by Vial *et al* wherein they analyzed the intracellular dehydrogenase activity [143]. To differentiate truly internalized NDs from surface bound-membrane trapped NDs, low concentrations of NDs were used with short incubation time. There was no significant cytotoxicity reported by the authors.

It is also important to study the hemocompatibility of ND as it will be majorly administered through blood. A research set up by Li *et al* showed negligible haemolytic and thrombogenic activity by oxidative treated HPHT NDs [144]. To further confirm and validate hemocompatibility, CCK-8 viability assay was performed on endothelial cells (HUVEC). Thus, it provides air-tight data on hemocompatibility. As stem cells form an important part of future cellular therapies and diagnostics, it is important to know the effects of ND labelling on them. Embryonic stem cells showed DNA repair protein, a possible indication of DNA damage. However, downstream markers were not affected [145]. Functionality of adipose mesenchymal stem cells was studied by Blaber *et al* [146]. There was no indication of intra cellular protein abnormality, stress or toxicity. The differentiation capacity indicated by CD marker expression remains unaltered. Compared to other emerging carbon-based carriers like CNTs, grapheme oxides etc, NDs are the most promising in terms of biocompatibility. Although immediate cytotoxicity is not observed its long-term studies have to be conducted.

In vivo studies have been done on smaller animals. *Caenorhabditis elegans* was labeled and imaged using fluorescent NDs. It did not affect the reproductive capability or longevity to the worms [147]. Mice studies have also been conducted to study toxicity and the conclusions are varied. Acute toxicity studies in Kun Ming mice were conducted by Zhang *et al* [148]. After intratracheal instillation, NDs were mainly distributed in the lungs. Further histological studies were evidence for lung, liver and kidney toxicity in a dose-dependent manner. While another study by Yuan *et al* with ND exposure of 1 mg kg^{-1} of mice, showed there were no adverse effects on the lungs after intratracheal instillation [21]. Further it was observed that post exposure 1–28 d; alveolar macrophages cleared the NDs from the bronchi to trachea and excreted via the mucociliary system. Such contradicting results is an indication that more studies need to be conducted *in vivo* and better 3D *in vitro* models need to be developed to screen the effects of NDs.

It is observed that the NDs are largely non-toxic, especially when compared to other carbon-based NPs like CNTs. The uptake mechanism is dependent on the surface manipulation performed on the ND. The most robust results on uptake mechanism is by clathrin-mediated pathway and macropinocytosis. The versatility it offers to make surface chemical changes, large scale manufacturing and long-term stability are big advantages to pursue more research on NDs on research and industrial scale. Although there are studies which indicate that NDs itself may have a role in the anti-cancer effect, such arenas need to be further studied and deemed safe. Cytotoxicity studies *in-vitro* largely is in favour of NDs, as their interaction at cellular and molecular levels is minimal. However, there is a lack of robust information from animal models. There have been fewer *in vivo* studies and have contradicting results. Although there is a huge research gap to understand the functionality and its long-term effects, NDs are promising drug carriers, especially for drug resistant cancers.

6. Biomedical applications of NDs in cancer research

Recently, there has been an increase in interest in biological and medical uses for NDs in the realm of cancer research (figure 5). This is because NDs offer a unique combination of features. ND are far superior to other nanomaterials for biomedical applications due to their biocompatibility, low cost, scalable production, negligible toxicity, chemical inertness of the diamond core, chemistry of the ND surface, and bright and robust fluorescence resistance to photobleaching [30]. Lately, studies have focused on biomedical applications such as drug delivery and bioimaging approaches of NDs [149]. NDs possess several tunable properties such as size and structural alterations. Synthesis methods and purification techniques primarily affect the surface chemistry of the NDs [150]. For instance, the diamond core for pristine NDs is usually coated with non sp^3 carbon. The structure of these carbon outer shells can be considered as onion-like shells or could exhibit an amorphous nature [151, 152]. The core diamond for NDs can be produced by any suitable method based on the application or size required, however, the surface modification and functionalization are dependent on the purification methods as mentioned by Krueger and Lang [76]. Different methods for the synthesis of NDs can also influence the biomedical applications like the chemical vapour deposition technique for the synthesis of NDs in biological coatings, HPHT for imaging applications and detonation techniques for imaging or drug delivery approaches [31]. Their small size (5–10 nm) and low cytotoxicity make them a suitable candidate for targeted drug delivery and gene delivery applications [153]. The drug release capacity of the NDs is affected by their

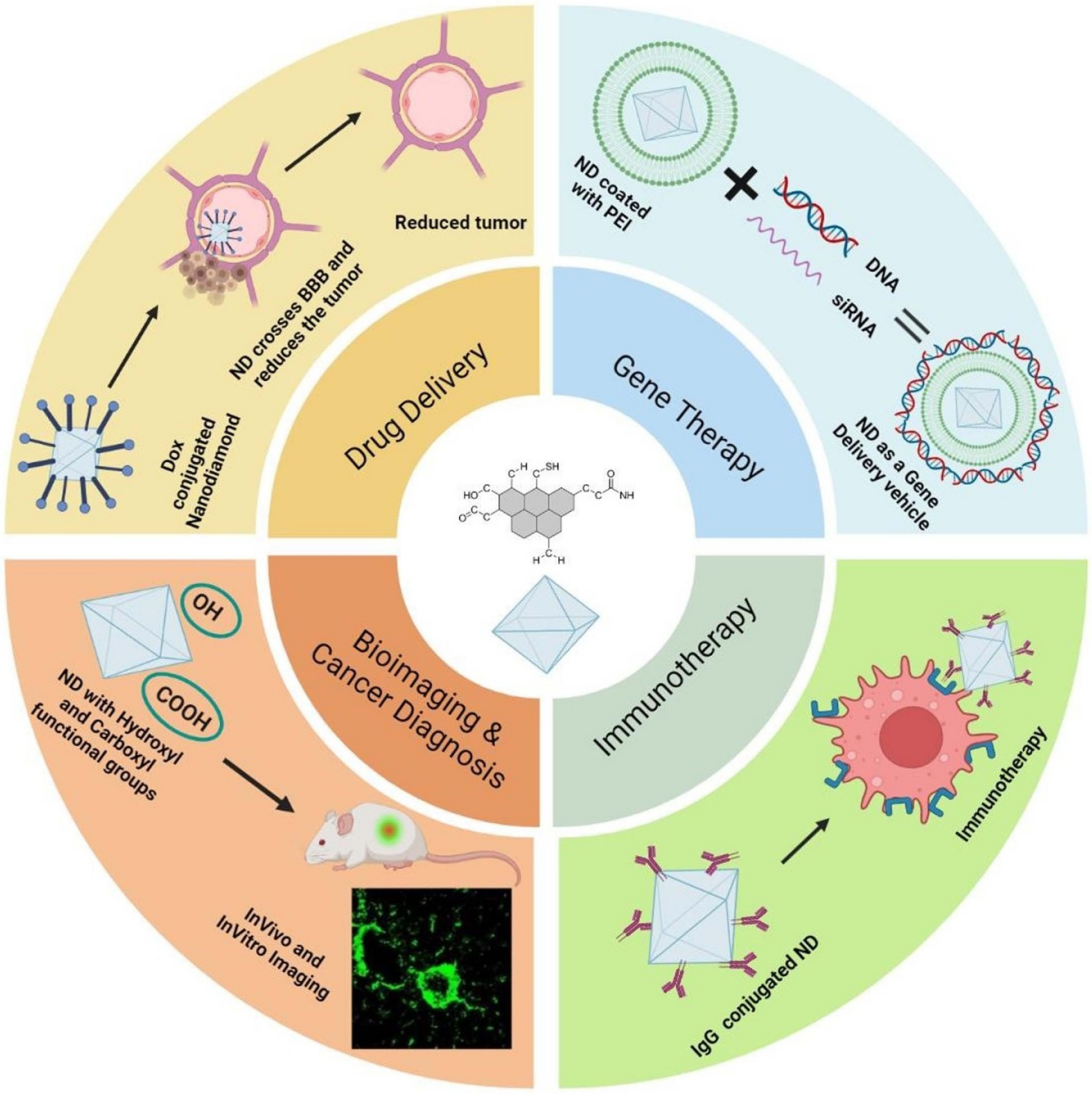

Figure 5. Various applications of ND in different biomedical aspects of cancer research such as drug delivery, gene therapy, immunotherapy, bioimaging and diagnosis.

sizes. The smaller the size, the larger the surface-to-volume ratio and as a result the drug loading potential increases [154]. Smaller size NDs of about 2–5 nm can be prepared via several common synthesis methods like detonation, and chemical vaporization [51, 155] while the HPTP method can be used to synthesize NDs of sizes ranging from 1–200 nm [156].

Under certain circumstances, DNDs tend to form agglomerates within the biological system thus losing their potential, therefore overcoming this issue by deagglomeration of NDs becomes a mandate. The primary source of aggregation is known to be oxygen-containing functionalities on DND surfaces and metal contaminants such as iron. The presence of functional groups comprising oxygen influences the surface potential, resulting in the aggregation of NDs through electrostatic forces with an average crystalline size of 4–5 nm [157]. The aggregates contain incombustible metal and oxide impurities that must be eliminated before the DNDs can be used. One of the effective approaches suggested by Korobov *et al* was monitoring the aggregation state or dispersity of powders or gels before the preparation [158]. The reproducibility or the efficacy of the NDs deteriorates in the complete absence of dispersity. Differential scanning calorimetry and x-ray diffraction techniques were used by the authors to monitor the dispersity from time to time during preparation. Santana *et al* proposed a bead-assisted sonic disintegration and carboxylation approach as another effective method to reduce the detonated ND aggregate sizes [159]. Considering all the unmatched advantages of different NDs, it can be concluded that they can be used as a promising potential candidate for several biomedical applications like chemotherapeutic drug delivery, immunotherapy, gene therapy biomedical imaging, development of biosensors and many more.

6.1. Drug delivery

An individual ND NPs surface is intricate and has a variety of surface moieties that enable conjugating molecules of interest. Through adsorption, covalent or non-covalent chemical immobilisation, or another method, the surface can be further altered with biologically active compounds [160]. NDs make drug distribution possible because of their quick passage across living cells' cell membranes [38, 74, 161–163]. NDs have shown promising results in cancer theranostics by functioning as drug carriers to boost a drug's therapeutic efficacy in comparison to traditional chemotherapy, as well as contrast agents for tumour detection [164, 165]. NDs have been utilized to circumvent the efflux and resistance of multidrug-resistant transporters, which prohibited the use of other NPs. For instance, epirubicin demonstrated approximately 10 times greater therapeutic efficiency when delivered via NDs as opposed to liposomes, which encountered high levels of rejection and resistance by efflux transporters in cancer cells [136]. It is a well-known fact that anticancer medications are prone to induce oxidative stress in patients with cancer being treated with chemotherapy [166]. Another beneficial aspect of NDs particularly helpful when utilizing them as drug delivery machinery is their well-documented antioxidative potential both *in vitro* as well as *in vivo*, wherein NDs depending upon their surface chemistry can quench ROS and scavenge free radicals [167]. Particularly, because of their thiol moiety, NDs have excellent antioxidant characteristics and may scavenge free radicals as well as reduce and chelate transition metal ions [168]. Carboxylation has been known to improve the antioxidant activity of DNDs further, which also has an effect on changes in the hydrogen bond strength in water [169].

It is noteworthy to mention that the BBB shields the neurons of the central nervous system, and serves as a diffusion barrier essential for protecting normal brain function by impeding most compounds from transiting from the blood to the brain. Hence, it is essential that the drug carriers themselves are biocompatible with the neural machinery and do not interfere with the therapeutic purpose if drug delivery methods are to be used to boost therapeutic efficacy. BBB presents a considerable barrier for neuro therapies because of resistance and ineffectiveness, which can result in negative effects [170]. Moreover, with brain dysfunction, there is a larger overexpression of efflux transporters, such as P-glycoprotein, a crucial ABC efflux pump in the BBB, which appears to weaken the BBB [171]. In such circumstances, NDs might be able to enter the brain and administer neuro medicine effectively, hence reducing the negative consequences linked to poor bioavailability [172]. People with brain tumours like glioblastomas have a dismal prognosis because of the BBB, and only have about a 1.5 year survival rate. Doxorubicin (DOX), a cornerstone medication for treating systemic neoplasms, has not been widely used to treat brain cancers due to its limited BBB penetration [173]. However, DOX, with the help of NDs, can not only penetrate the BBB to reach brain tumours but also become more effective. In a study by Xi *et al* convection-enhanced administration of an ND-DOX combination was demonstrated for the treatment of glioblastoma [174].

Equilibria studies between aqueous solutions of doxorubicin or polymyxin B with NDs were conducted by Mochalin *et al* under isothermal conditions for NDs having polyfunctional and aminated surfaces. Although both the drugs showed absorption that followed the Langmuir isotherm, the characteristics of adsorption, such as the maximal monolayer capacity and binding strength, showed a considerable dependence on the ND surface chemistry [175]. Surface modification of NDs was done by oxidation in air at a very high temperature 425 °C–430 °C for two hours. As a result of the purification, the groups containing oxidized NDs showed less heterogeneity both geometrically and chemically thus fitting the Langmuir model for data interpretation better.

Similarly, in another study, the kinds of surface groups on the primary 4 nm NDs manufactured by the NanoCarbon Research Institute were thoroughly investigated by Paci *et al* [176]. In numerous *in vivo* experiments, these NDs have been employed successfully as Dox drug carriers, and the ND-Dox complexes have shown extremely high efficacy in the treatment of cancer [175, 177, 178]. Preliminary research showed that the activity of Dox molecules was diminished while they bound to the ND surfaces in such a way that the cytotoxicity of Dox may be reduced potentially. To better understand the distinct chemical-physical characteristics of the ND facets, several modelling/simulation techniques have been used to study the interactions between the ND surface and diverse kinds of medicinal chemicals [179, 180]. The scientists quantitatively characterised the functional groups on the surface of these NDs using a variety of experimental and computational methodologies. They concluded that the groups are amphoteric and contain significant amounts of pyrones, phenols, and sulfonic acid groups. Phenols and pyrones were consistent in the presence of graphitic surfaces created during bead milling [181]. In the ND purification procedure, sulfuric acid is utilised, and this produces the sulfonic acid groups. Schrand *et al* determined that the presence of pyrones is the source of the positive surface potential of these NDs, which is compatible with a discussion of the origin of positive zeta potential in NDs [163].

In conclusion, ND-based anticancer medicines show considerable potential and open a variety of novel, intriguing approaches. For instance, recent developments in the induction of vascular leakiness may make it possible to deliver larger quantities of chemotherapy to tumours at the beginning of tumorigenesis, before the EPR effect has fully developed.

By using ND platforms, significant progress was made in the delivery of weakly water-soluble anticancer medicines. The large and customizable surface of ND allows the adsorption and sustainable release of anticancer medicines in response to pH changes and other signals. ND-drug adsorption complexes have demonstrated considerable promise in eradicating drug-resistant cancer, avoiding drug efflux, and minimising the side effects of anticancer medications. Moreover, ND-drug adsorption complexes have been employed in combination therapy as a component of effective drug cocktails to treat multidrug-resistant tumours and battle migratory CSC-caused metastases [182]. The ability to circumvent the resistance of efflux transporters seems to be an advantage more notably connected with NDs than with other NPs. Such studies have repeatedly created the groundwork for exploiting this ability to also treat neurological illness (table 3).

6.2. Gene therapy

Due to the lack of reliable vector systems that can carry nucleic acids to patients for treatment, gene therapy is still a challenge. Though viral vectors are effective but they may be risky for routine therapeutic usage. While synthetic non-viral vectors are inherently safer, they are not yet effective enough to be used in clinical studies. With enhanced synthetic non-viral vectors built upon well-established platform technologies and a complete understanding of the obstacles to effective gene transport and expression (transfection) relevant to therapeutic applications of interest. Gene therapy can be used in the future with reduced risk and increased effectiveness. Nanocarriers must protect against DNA deterioration once they have entered the cell, yet they frequently do not provide a sufficient release of DNA from endosomal compartments. If the gene payload fails to get released from the endosomes, it is transported to the lysosomes, where it is broken down by the many nucleases, which may result in transfection failures. However, scientists created potent ND for secure and reliable replacement for gene delivery [188]. NDs have the capacity to effectively and safely introduce DNA coding for the production of different proteins to the cells in a culture when coated with the polycationic PEI [178]. An earlier study by Alhaddad *et al* to determine the possibility of introducing nucleic material to treat Ewing sarcoma [189]. NDs were used as a cell-targeting bifunctional device that served as both a medication delivery system and a fluorescent label. While delivering siRNA to human cell lines derived from Ewing sarcoma tumours, NDs were employed as the delivery vehicle. Conventionally chemotherapy, radiation, and surgical resection are all used to treat this cancer. However, besides the traditional treatments, novel techniques based on the targeting of the EWS-Fli1 junction at the mRNA level by antisense oligonucleotides (ODN) or siRNA that block the EWS-Fli1 gene have also been developed [190]. siRNA was demonstrated to cause the cleavage of EWS-Fli1 mRNA and to reduce both tumour growth and EWS-Fli1 expression *in vitro* and *in vivo* conditions. Fluorescent NDs were employed to test their ability to (a) block EWS-Fli1 expression in cell culture at both the mRNA and protein levels, (b) bind to siRNA and (c) allow for its cellular absorption. This vector exhibit advantages compared to the commonly used transfection agent Lipofectamine in terms of effectiveness at reducing EWS-Fli1 expression as well as cell toxicity in serum-supplemented media.

Gu *et al* demonstrated the use of NDs as a delivery vehicle for the administration of G9a inhibitors for hepatocellular carcinoma therapy [191]. Histone methyltransferase G9a, commonly referred to as euchromatic histone-lysine N-methyltransferase 2 (EHMT2), is primarily in charge of the dimethylation of histone H3 lysine 9 (H3K9) [192–194]. G9a has been found to play an important role in the progression of tumours in various cancers, including HCC [193–195]. Furthermore, it was discovered that the greater the G9a expression, the worse the outcomes. These studies show that G9a has a high clinical significance as a possible therapeutic target [196]. In this study, UNC0646 was used as it potentially inhibits the activity of G9a by competitively binding in the substrate peptide-binding site [197, 198]. They demonstrated that in a controlled environment, ND-UNC0646 complex and UNC0646 both showed comparable *in vitro* cell-killing efficacy; however, over the long run, ND-UNC0646 displayed a greater sustained efficacy. Moreover, ND-UNC0646 has shown a greater ability to decrease H3K9 methylation by inhibiting methyltransferase activity as well as a greater ability to inhibit invasion. Further, during *in vivo* studies it was discovered that ND-UNC0646 complexes are able to maintain the biological functionality of the original G9a inhibitor meanwhile exhibiting improved tumour inhibitory activity.

The average diameter of NDs is around 5 nm, and they have a low dispersity index and a sizable surface area. Due to their nanoscale size and van der Waals forces, NDs have a tendency to self-agglomerate, which contributes to their poor stability in a range of media. In actuality, NDs alone in water suspension were discovered to have a mean size of 89 nm. Therefore, NDs must be functionalized or attached to additional substances, typically polymers for gene delivery Karami *et al* [81]. Hence, it stands to reason that NDs would have exceptional compatibility with biological systems, making them a promising alternative for biomedical applications. Although encouraging, these results only serve as a proof-of-concept, and additional studies in animal models are necessary to confirm the observed potential of NDs for gene delivery.

Table 3. List of chemotherapeutic drugs used in association with nanodiamonds and their outcomes.

Sl. No.	Composition	Components	Type of carcinoma	Results	References
1.	NDs	Purvalanol A, 4-hydroxytamo- xifen	Ductal or lobular carcinoma	NDs increased the drug diffusion in water and reduced the drug cytotoxicity. It promotes sustained release of the drug to target cancer cells.	[183]
2.	ND-embedded polymeric microfilms	Doxorubicin	• Soft tissue and bone sarcomas • Acute lymphoblastic leukemia • Acute myeloid leukemia • Ovarian carcinomas • Hodgkin lymphoma • Non-Hodgkin lymphoma • Adenocarcinomas • Anaplastic carcinoma • Neuroblastoma	Sustained release of the drug is achieved by conjugation of ND-DOX. DOX-NDs patch are used in pre- and postoperative therapies for localised therapeutic release, tumour apoptosis before surgical intervention, or lowering the risk of cancer recurrence post-surgery	[184]
3.	DOX and TAT conjugated to NDs surface through carbodiimide coupling	Doxorubicin	Neuroblastoma	TAT-ND-DOX exhibits a greater cytotoxic effect and improves translocation through the membrane of C6 glioma cells.	[185]
4.	NDs covalently conjugated with PEG-4000	Doxorubicin	Hepatocellular carcinoma	Dox is delivered to HepG2 by clathrin-dependent endocytosis, increase of uptake half-life of ND-PEG-DOX compared to free DOX.	[126]
5.	ND conjugated with antibody cetuximab	Paclitaxel	• Bronchogenic carcinoma • Kaposi sarcoma • Ovarian carcinomas • Ductal or lobular carcinoma	Nanodiamonds decorated with antibodies delivered paclitaxel to human colon cancer cells. Paclitaxel inhibits microtuble delivery in cancer cells and causes mitotic failure thus suppresses the tumor growth.	[120]
6.	ND conjugation with drug via reversible binding	Daunorubicin	• Acute lymphoblastic leukemia • Acute myeloid leukemia	Daunorubicin binding with nanodiamond reduces effective drug dose as well as drug toxicity. Side effects of the drug also get potentially reduced when it is used in conjugation with nanodiamond.	[186]

(Continued.)

Table 3. (Continued.)

7.	ND as carriers	Epirubicin	• Ductal or lobular carcinoma • Urothelial carcinoma	Epirubicin when delivered via nanodiamonds as carriers, there was a sustained release of the drug only after the cancer cells internalized with the complex and made the microenvironment acidic. The intracellular acidic pH and proteins together triggered the drug release.	[135]
8.	ND-drug complex	Mitoxantrone	• Ductal or lobular carcinoma • Acute leukemia • Lymphoma • Adenocarcinoma of the prostate	ND-MTX complex promoted sustained drug release to combat the treatment failure caused by chemoresistant breast cancer cells.	[187]
9.	ND as carriers	Citropten	• Colon carcinoma • Squamous cell carcinoma	Nanodiamond carriers with citropten alters the activity of the actin filaments involved mitosis. Thus, the ND-CIT complex inhibits the cell proliferation of rapidly dividing cancer cells and minimizes the toxicity on healthy tissues.	[137]

6.3. Immunotherapy

An important hallmark of cancer is to evade the body's immune system. A tumor microenvironment (TME) contains cancer cells as well as stromal components like blood vessels, fibroblasts, and infiltrating immune cells [199]. Nanotechnology-based immunotherapy designed to target tumor cells predominantly employs two approaches which includes immune stimulation and immune suppression. In immune stimulation, NPs are engulfed by phagocytic cells thereby eliciting an immunological response either through antigen delivery to specific cells or through antigen presenting cells [200]. FNDs faceted framework allows the conjugation of immunostimulatory agents. Innate immune cells readily engulf, stimulate FNDs, and pro-inflammatory responses without disrupting the cell viability. Innate immune cells such as monocytes and natural killer cells are primary players in cancer immunosurveillance as they eradicate developing tumor cells. When these cells are activated, adaptive immune cells are recruited furthering the immune response [201].

When IgG molecules are attached to the surface of FND, they make it easier for FcR to bind. This happens because the Fc region of IgG is exposed, which activates immune cells that have FcR and causes FND to be taken up. Recently Kelly *et al* reported the preparation of glycidol coated FNDs (gFND) conjugated with IgG (IgG–gFND) towards developing an effective immunotherapy platform against cancer [202]. The authors demonstrated the antibody-conjugated FND (IgG-gFND) to be biocompatible, besides retaining the excellent photostable NIR fluorescence, and biological properties of antibodies. The gFND significantly reduced non-specific cellular FND uptake, whereas the IgG antibody conjugation of gFND allowed for a significant increase in FND uptake by both monocytes and NK cells. Validation of ND immune cell targeting in a tumor model and an immune cell co-culture system, revealed the immune cells to be preferentially absorbing IgG-gFND as a result of which significant immune cell activation occurred with no compromise in immune cell viability. Essentially, in contrast to uFND, which migrated to the liver and kidneys after intratumoral injection, IgG-gFND remained at the tumor site. As a result, antibody-conjugated FNDs could potentially be used as an immunotherapy

strategy with 'track and trace capabilities' to promote targeted antitumor activity while minimizing systemic toxicities.

As in immune-suppression, when breast cancer patients were administered with a NP colloidal suspension Abraxane consisting of serum albumin NPs bound paclitaxel, myelosuppression and reduced incidence of neutropenia was observed [203]. Researchers have demonstrated NDs ability to reverse both systemic and tumor-induced immunosuppression, thereby avoiding chemoresistance [204]. Yuan *et al* recently reported the intravenous delivery of doxorubicin-polyglycerol-nanodiamond conjugate (ND-DOX) to mammary carcinoma mice model, which demonstrated efficacious therapeutic potency and lower toxicity than using DOX alone [138]. Utilizing ring-opening polymerization of glycidol via hydroxyl groups located on the ND surface, polyglycerol was grafted on hydroxylated ND. Following that, partial hydroxyl groups on ND-PG were converted to hydrazine groups in order to conjugate Doxorubicin on the ND-PG. P-gp and IL-6, which have been shown to be key mediators of Doxorubicin chemoresistance in 4T1 cells, were not significantly upregulated by Nano-DOX. Furthermore, Nano-DOX was demonstrated to inhibit tumor-derived granulocyte-colony stimulating factor (G-CSF) induction and tissue filtration of MDSCs, which are the primary effectors of cancer-associated systemic immunosuppression. Overall, Nano-DOX as a chemotherapeutic agent in nano-form was argued to possess distinct biochemical properties from its free form, which could be used to combine chemotherapy and immunotherapy for better treatment of cancer. More recent studies reported that HPHT-synthesized NDs at the cellular level neither caused pro-inflammatory cytokine production nor activated transcriptional factors which do not interfere with macrophage functionality [205].

Dendritic cell (DC)-based immunotherapy, which is a potential therapeutic approach against the deadliest primary brain tumor Glioblastoma (GBM) is primarily impaired due to GBM-induced immunosuppression [173, 206–208]. This immunosuppression can be reverted by damage-associated molecular patterns (DAMPs) emission. Following this trajectory, Li *et al* investigated the potential of overturning the GBM-mediated immunosuppressive microenvironment by carrying out a DC mediated delivery of doxorubicin-polyglycerol-nanodiamond composites (Nano-DOX) [209] (figure 6(A)). Nano-DOX, which was previously reported to be a potent DAMPs inducer, could riggering an enhanced anti-GBM immune response resulting in effective immunotherapy against GBM-induced immunosuppression. Nano-DOX-loaded DC were demonstrated to be functionally viable and capable of releasing cargo drug to co-cultured GBM cells (GC). Not only did Nano-DOX-treated GC emit a high level of DAMPs, but it also released antigens, which enhanced the stimulation, acquisition, and presentation of GC-derived antigen in DC co-cultured with GC and Nano-DOX. Co-culture with GC and Nano-DOX consistently activated mouse bone marrow-derived DC, which stimulated mouse spleen-derived lymphocytes, and suppressed co-cultured GC. The authors through their work demonstrated the effectiveness of using DC-mediated Nano-DOX delivery to stimulate GC immunogenicity thereby generating a strong anti-cancer immune response in the Glioblastoma tumors [209].

As immunostimulatory agents against infectious diseases and cancers, Cytosine-phosphate-guanine (CpG) ODNs have undergone thorough rounds of FDA-approved phase I–III clinical trials [203, 211, 212]. However, less cellular uptake and prompt clearance of unbound ODNs became a major hindrance to the study. Zhang *et al* reported a three-fold increase in the cellular uptake when ODNs were delivered using a form of functional ND-based agent (figure 6(B)). This study incorporated poly (D-lysine) (PDL)-coated functional NDs (fNDs) for *in vitro* and *in vivo* ODN delivery. CpG-fNDs, when internalized, significantly induced cytokine production by binding to TLR-9 in the lysosomes. Due to their porous structure, fNDs could protect and provide uninterrupted release of CpG causing immunomodulatory activities to last up to 2 d in mouse models and 3 d at the cellular level. Consequently, significant tumor suppression was observed. Overall, the high biocompatibility and lower cost make these fNDs a potential candidate to be explored in cancer immunotherapy in humans [210].

In immune cell activation, programmed death-ligand 1 (PD-L1) and programmed cell death-1 (PD-1) are critically important negative regulators. PD-L1 are ubiquitously expressed in various tumors with an ability to suppress anti-tumor immune response facilitated by tumor permeating cytotoxic T lymphocytes with PD-1 receptor [213]. Therapeutic approaches involving blockage of PD-L1/PD-1 interaction between T lymphocytes and cancer cells have demonstrated remarkable potency for promoting anti-tumor immunity and treating malignant diseases [214, 215]. The expression of PD-1 was observed in tumor-associated macrophages (TAMs) which are the most plenteous leukocyte in the TME [216]. TAMs account for almost 50% of the tumor mass in specific tumors having intricate interactions with cancer cells [217–219]. Zhen *et al* in their study demonstrated the potency of Nano-DOX to cause the release of high mobility group box 1 protein by stimulating tumor cells and concurrently upregulating PD-1s in TAMs and NF-κB-dependent PD-L1 in tumor cells. They also showed the therapeutic synergy exhibited by BMS-1, a PD-1/PD-L immune checkpoint inhibitor with Nano-DOX as it blocked PDL-1 induced by Nano-DOX in tumor cells, further

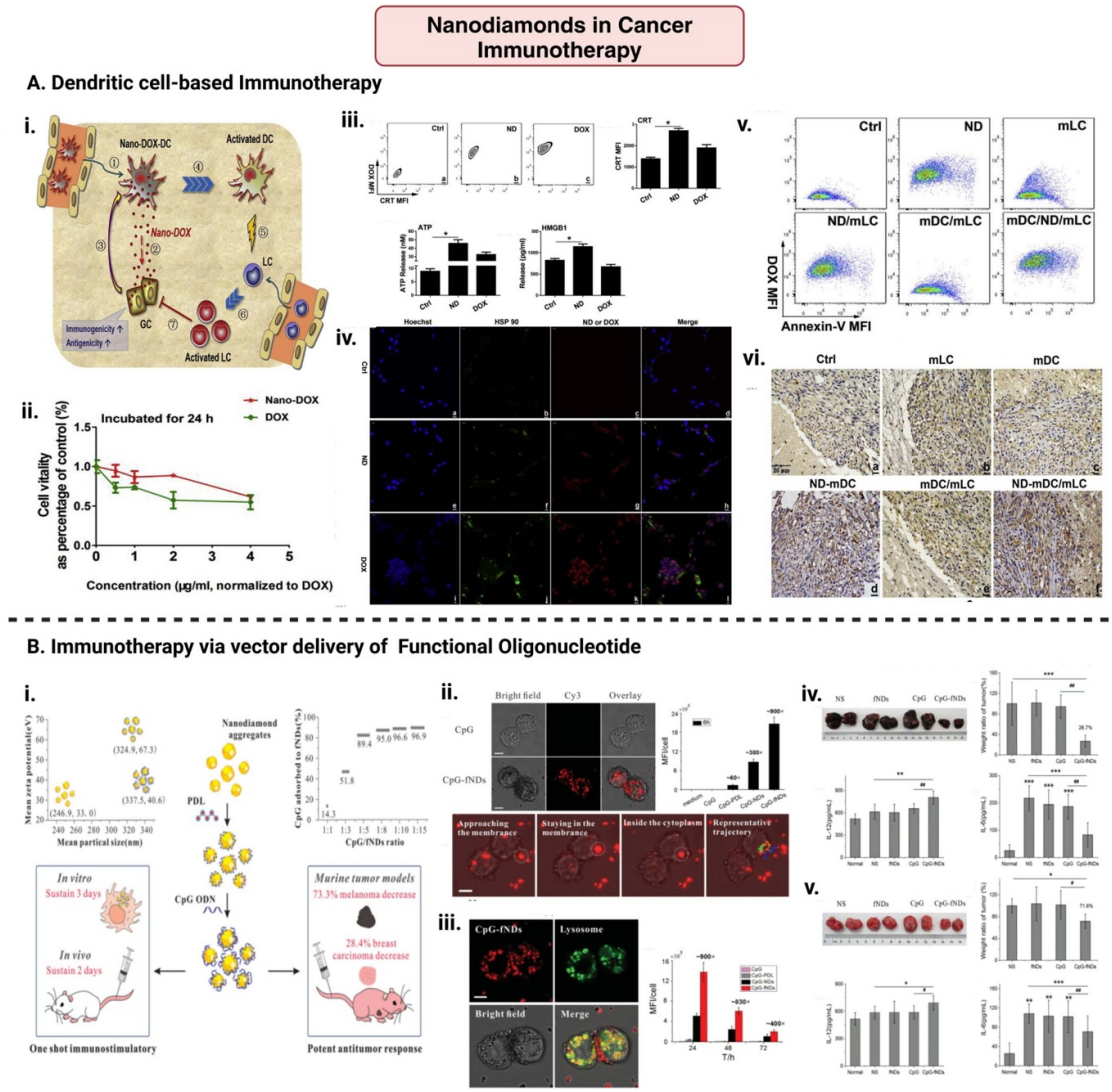

Figure 6. Application of Nanodiamonds in Immunotherapy. (A) Dendritic cell-based immunotherapy involving nanodiamonds. (i) Schematic of dendritic cell-mediated delivery of doxorubicin-polyglycerol-nanodiamond (Nano-DOX-DC) composites eliciting enhanced anti-cancer immune response in glioblastoma. (ii) Assessment of cell viability of Dendritic cells treated with Nano-DOX or DOX for 24h using CCK-8 test. (iii) Nano-DOX promotes DAMPS emission and MHC-I expression in glioblastoma cells (GC) observed using FACS analysis of CRT immunofluorescent staining (top), and by measuring supernatant levels of ATP and HMGB1 using Chemiluminescence and ELISA. (iv) Confocal microscopy. Blue fluorescence indicates nuclear counterstaining; green fluorescence indicates immunostaining staining of HSP90; and red fluorescence comes from Nano-DOX or DOX. (v) Apoptosis of GC treated with with Nano-DOX detected through FACS analysis of surface-exposed annexin v immunofluorescent staining. (vi) Apoptosis in mouse orthotopic GC xenografts observed through immunohistological staining which detected caspase-3 activity in all groups that received Nano-DOX-mDC. Reprinted from [209], Copyright (2018), with permission from Elsevier. (B) Nanodiamonds as delivery vectors of functional oligonucleotides for cancer immunotherapy. (i) Overall strategy involving the preparation of fNDs and their immunomodulatory activity for cancer therapy. (ii) Confocal images depicting the cellular uptake of Cy3-CpG-fNDs by RAW264.7 cells after 6 h incubation with 5 μg ml^{-1} Cy3-CpG and 50 μg ml^{-1} Cy3-CpG-fNDs. Scale bar: 5 μm (left). The mean fluorescence intensity (MFI) inside the cells (right). (iii) Confocal images corresponding to co-localization of CpG-fNDs (red) and lysosomes (green) in RAW264.7 cells incubated with 50 μg ml^{-1} Cy3-CpG-fNDs for 6 h. Scale bar: 5 μm (left). MFI inside the cells at 24, 48, and 72 h after the initial 6 h incubation (right). (iv) Potent antitumor effects of CpG-fNDs in murine tumor (B16-F0) model in terms of weight ratios of tumors (top), and the serum levels (mg ml^{-1}) of IL-12 and IL-6 analyzed through ELISA (bottom). (v) Potent antitumor effects of CpG-fNDs in murine tumor (4T1) model in terms of weight ratios of tumors (top), and the serum levels (mg/mL) of IL-12 and IL-6 analyzed through ELISA (bottom). [210] John Wiley & Sons. © 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

stimulating anti-tumor immunity mediated by TAMs [220]. (Table 4) provides a summary of application of NDs for immunotherapy applications against cancer.

6.4. Bioimaging and cancer diagnosis

NDs with their truncated octahedral structure are known for their excellent biocompatibility which have been demonstrated through *in-vitro* assays and animal-derived models [221]. Surface modification by means of addition of hydroxyl and carboxyl functional groups enable NDs to electrostatically interact with several molecules. This has important implications when integrating NDs with fluorophore target proteins which produces fluorescence in cells

Table 4. List of nanodiamonds associated with immunotherapy.

Sl. No	ND type	Cancer type	Immunotherapeutic Load	Characteristics	Mechanism of action	References
1	CpG-fNDs	Melanoma, breast carcinoma	CpG ODNs	Nanodiamonds, coated with poly (D-lysine) (PDL) are electrostatically linked with immunostimulatory nucleic acids such as unmethylated cytosine-phosphate-guanine (CpG). These complex forms spongy porous structures. The uptake efficiency of CpG highly increased due to its association with functionalized NDs (fNDs)	Due to their porous structure, CpG-fNDs could significantly induce cytokine production by binding to TLR-9 in the lysosomes for 2–3 d.	[212]
2	IgG-fND (ImmunoglobulinG—Fluorescent nanodiamond)	Breast cancer	IgG (Antibodies)	FND's possess nitrogen vacancy centres and emit photostable fluorescence of approximately 700 nM wavelength in the near infrared region on the photo spectrum. Their larger and readily modifiable surface area makes them accessible for the conjugation of various molecules. Conjugation of IgG molecules on the surface of FND could expose the Fc regions of the IgG for FcγR binding and thereby activate FcR-expressing innate immune cells and/or the uptake of the FND.	After treatment, a stable surge in IL-12 and a drop in IL-6 levels in both tumor models demonstrated the anti-tumor function of CpG-fNDs through observed immunomodulatory effects.	[201]

(Continued.)

Table 4. (Continued.)

3	Doxorubicin-polyglycerol-nanodiamond conjugate (Nano-Dox)	Glioblastoma (GBM) Triple negative breast cancer (TNBC)	Doxorubicin-polyglycerol	Nano-DOX with a hydrodynamic diameter of 83.9 ± 32.3 nm has its core coated with polyglycerol to which the DOX is attached. On the breakage of this bond, the DOX is released in the lysosome's acidic environment thereby reaching the nucleus. It Induces immunogenic cell death in cancer cells.	In GBM, FND-conjugated antibodies are also observed to selectively target innate immune cells and suppressor cells in tumor-associated macrophages and myeloid-derived suppressor cells. The cytostatic action of Nano-Dox in 4T1 cells (Triple negative breast cancer mouse cell line model) was demonstrated. It did not upregulate chemoresistance mediators like IL-6 and P-gp in 4T1 cells The Tissue infiltration and expression of oncogenic immune-suppression effectors such as myeloid-derived suppressor cells (MDSCs) and tumor-derived granulocyte-colony stimulating factors was downregulated.	[138, 208, 219]
4	NDs-PDL-RNase	Cervical cancer (HeLa), breast cancer (MCF-7)	RNase A	Poly-D-Lysine (PDL) enables the association between proteins and NDs and facilitates cytosolic desorption of proteins. The complex remains in the cytoplasm and escapes protein degradation.	This induced apoptosis in about 70% of HeLa and MCF-7 cells by degrading the RNAs	[220]

upon excitation proving helpful in bioimaging applications. Bioimaging has played a crucial role in cancer diagnosis lately. Several bioimaging techniques such as magnetic resonance spectroscopy, MRI, PET scan, single-photon emission computed tomography and optical imaging techniques are used for early

detection of cancer cells [222]. In a recent study by Hu *et al* the cellular internalization of the NDs complexed with red fluorescent proteins (NDs–RFP complex) was observed using a red fluorescence signal when incubated with Hela cells [223] (figure 7(A)). Similarly, respective fluorescence was observed when the cells were incubated with yellow, green, and cyan fluorescent proteins. Additionally, the zeta potential of the NDs were reported to decrease from 31 mV to 12 mV due to RFP absorption, indicative of instability resulting from reduction in stabilizing repulsions potentially leading to aggregation of the ND particles [224]. RFP was also observed to elevate NDs absorption capacity. Subsequently imaging through a total internal reflection fluorescence microscope to track the cellular internalization of a single NDs-RFP complex was effectively carried out revealing that the retention of the NDs–RFP particles on the cell membrane lasted for about 90 s before it moved into the cytoplasm [223]. Imaging studies also revealed that only about half of internalized NDs enter the endosome/lysosome route, while the rest avoided merging with the early endosome. As a result, substantial levels of NDs-protein were observed in the cytosol [225]. This study laid the basis for leveraging the unique properties that allow NDs to be used as multimodal theranostic models as well as bioimaging bio-labels.

Other substances can also be integrated into FNDs to achieve efficient characteristics like better surface chemistry, non-cytotoxic, photostability, biosafety, higher biocompatibility, and intense multicolored fluorescence. FNDs incredibly facilitate an assemblage of biomedical purposes including cell imaging using NDs with light-emitting capacities as they possess an array of photoluminescent and spin characteristics [226]. Leung *et al* in their study, demonstrated the effective use of PEGylated BSA and RGD peptide integrated with carboxylated NDs (RGD-dcBSA-PEG-NDs) for *in-vitro* and *in-vivo* targeting and imaging of brain tumor [227]. The polymeric coating was found to protect the NDs from aggregation, maintaining better colloidal stability in various physiological environments. This discrete complex was observed to traverse the BBB model better than aggregated NDs. Moreover, the prepared RGD-dcBSA-PEG-NDs, unlike non-targeting NDs, can preferentially target the tumor location in U-87 MG carrying mice following systemic injection. Overall, this distinct ND system allowed for effective brain tumor visualization while causing minimum damage to other key organs [227]. Spectroscopic characteristics enable FNDs to be viewed background-free in tissue sections and facilitate a prolonged cell-tracking operation, especially in stem cells. FNDs with NV centers serve as excellent contrast agents for achieving super-resolution imaging through stimulated emission depletion [228]. Although many contributions have been made to the use of covalently conjugated NDs as cellular biomarkers, there are disadvantages in fluorescence bioimaging such as imaging probe degradation, biological toxicity, tissue light scattering, etc. An early study by Wu *et al* involved the FND-labelling of lung stem cells (LSCs) which were transplanted into mice to trace their engraftment and regenerative capabilities [229]. It was also observed that this labeling did not affect the differentiation and self-renewal properties in pneumocytes. FNDs have been employed to track slow-multiplying and stagnant CSCs that initiate tumors. Additionally, as FNDs are stable chemically and photo-physically, they are used for *in vivo* and *in vitro* identification and isolation of these cells. Overall, the authors concluded that it would be easier to understand CSCs *in vitro* and *in vivo* with the combination of an FND-based tracking scaffold and protein biomarkers [228]. Another study demonstrated that endocytosis enabled FND labeling of LSCs did not affect cellular functions. A week after intravenous injection, FND-integrated LSCs in mice could be tracked using FND combined with fluorescence time imaging microscopy (FLIM), immunostaining, and FACS. Using this labeling, the cells can also be quantitatively evaluated after transplantation [229].

Cancer detection at an early stage and treatment relies primarily on the imaging and detection of *in vivo* tumor growth. When it comes to tongue cancers, the tumor's thickness can be accurately determined by MRI based on RF-induced spin-relaxations which can also be applied in diagnosing breast, prostate, and rectal cancer. Due to its excellent spatial resolution and lack of ionizing radiation, MRI is essential for tracking cancer cells *in vivo*. Many MRI contrast agents are necessary to label cells, however, they either do not accumulate appreciably in cells or are not biologically compatible for translational investigations. The tumor tissue at each stage grows in size starting from 3–9 mm which could be observed by MRI along with integrating magnetic NPs to FNDs with increased contrast and resolution. In a recent study, cancer growth was tracked and morphologically analyzed in mice models using FNDs and Gadolinium (III) (FDG) magnetic molecule aggregates [165] (figure 7(B)). Interestingly, the NDG exhibited high relaxivity irrespective of field strength, which was previously unreported for gadolinium (III) [Gd (III)]-NP conjugates. Additionally, T1- and T2-weighted MR imaging allowed tracking of tumor progression of NDG-labeled flank tumors for 26 d *in vivo*, which is longer than had been reported with other MRI contrasting agents. The authors observed a 300-fold hike in Gd complex delivery in the cell without causing any cellular toxicity. Cell viability was also determined to be almost 90% along with an excellent MRI contrast compared to other carbon-based substances like CNTs and graphene [230].

Although ND-Gd (III) complexes have increased relaxivity, they have also been observed to possess

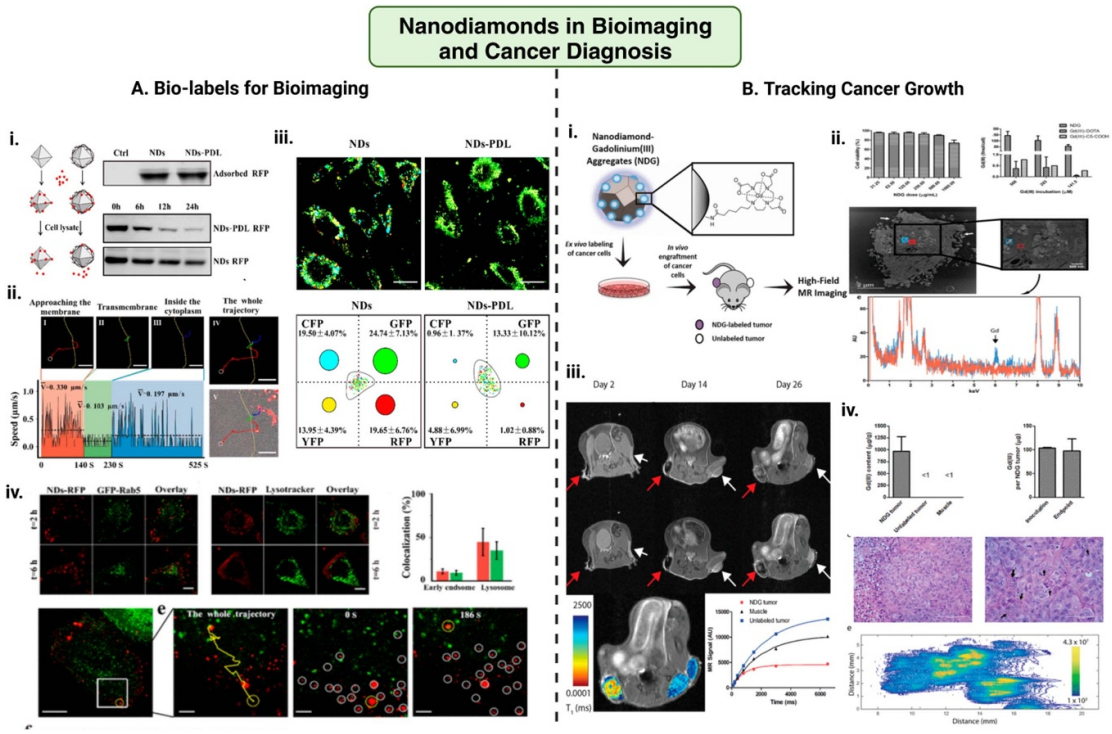

Figure 7. Application of nanodiamonds in bioimaging and cancer diagnosis. (A) Nanodiamond used as bio-labels for bioimaging. (i) Adsorption of RFP to NDs or NDs–poly-D-lysine (PDL) in PBS and the desorption of RFP from NDs or NDs–PDL in cell lysate. (ii) Live-cell imaging of NDs–RFP's internalization with a TIRF microscope revealing the dynamic internalization of NDs–protein complex. The trajectory of the internalization of single NDs–RFP particle comprised of the following stages and were delineated by different colors: (I) approaching the membrane, red; (II) staying on the membrane, green; (III) inside the cytoplasm, blue; (IV) the whole trajectory; (V) bright field. Scale bars = 10 μm. (iii) Confocal fluorescence imaging showing concurrent delivery of CFP, GFP, YFP, and RFP into Hela cells by NDs or NDs–PDL. Scale bars = 25 μm. (top. Quantitative analysis of fluorescence signals in Hela cells ($n = 20$). The ratio of non-overlapped signals from each type of protein was indicated in the corresponding quadrant, and overlapped signals were scattered near the origin of coordinates (Bottom). (iv) Colocalization of NDs–RFP with the endosome and the lysosome. Confocal fluorescence imaging showing the subcellular localizations of NDs–RFP and early endosomes (GFP–Rab5) or endosomes/lysosomes (LysoTracker Green) at the indicated time after NDs–RFP was added to Hela cells. Scale bars = 10 μm. (Top). Live-cell imaging of a single NDs–RFP particle (in the yellow circle). Scale bar = 10 μm. (Bottom left) Enlarged view of the square region in part d showing the whole trajectory, and the starting and ending points. White circles indicate other NDs–RFP particles. Scale bars = 2 μm. (Bottom Right). Reprinted with permission from [223]. Copyright (2017) American Chemical Society. (B) Nanodiamond used for tracking cancer growth. (i) Schematic describing the Nanodiamond–Gadolinium(III) Aggregates (NDG) for tracking cancer growth *in vivo.* (ii) Cell viability analysis showing that NDG is well-tolerated across a wide dose range (Top Left). Graphical representation showing NDG confers 300-fold improvement in cellular delivery of Gd(III) compared to Gd(III)–DOTA and Gd–C5–COOH (Top Right). STEM image of single cell after 24 h of incubation with NDG. Enhancing NDG aggregates seen inside the cell and being engulfed near the plasma membrane (white arrows) (Middle). EDX spectroscopy showing the Lα1 peak of gadolinium beingclearly observed in the spectrum for the region bearing NDG aggregates (teal) and not in the region of vacant cytoplasm (red). The Lα2 peak of gadolinium is also seen (Bottom). (iii) 7 T MR images of a SCID-beige mouse bearing a NDG-labeled xenograft and an unlabeled xenograft of MDA-MB-231 m-Cherry cells ($n = 5$, representative mouse shown). Images shown are of 2, 14, and 26 d after engraftment. NDG tumor is on the right flank (left in page, red arrows); unlabeled tumor is on the left flank (right in page, white arrows). (iv) Gd(III) content of tumors harvested at the 26 d end point ($n = 3$). The NDG tumors have high Gd(III) content of approximately 1 mg per g of tissue, while unlabeled tumors and muscle has negligible quantities of Gd(III) (Top Left). Gd(III) content in NDG tumors was compared between the inoculation time point and the 26 d end point, and on average, 95% of the Gd(III) remained within the tumor (Top Right). Hematoxylin and eosin (H&E) section of unlabeled tumor (40 × magnification) showing uniform, invasive neoplastic cells along with a region of central clearing indicative of necrosis, along with showing several mitoses indicative of a high proliferative rate (Middle Left), and the H&E section of NDG tumor (60 × magnification) showing a similar morphology to the unlabeled tumor but containing visible NDG aggregates within neoplastic cells and in the interstitial space (black arrows) (Middle Right). Spatial distribution of Gd(III) in a cross-section of the NDG tumor, quantified using laser ablation ICP-MS. Gd(III) is distributed throughout the section, with highest concentrations in the center (Bottom), Reprinted with permission from [165]. Copyright (2016) American Chemical Society.

less dispersibility in physiological media due unconjugated ND's limited hydrophilicity along with the highly hydrophobic bonds between Gd (III) and ND complex. In order to increase ND's dispersibility, Zhao *et al* in their study, incorporated hyperbranched polyglycerol (PG) particles covalently on the ND surface hydroxyl and/or carboxyl groups facilitated by ring-opening polymerization [231]. It was found that in addition to a better aqueous dispersibility (>4.5 mg ml^{-1}) and stability (>3 months) in PBS, PD grafting also added greater versatility via further surface functionalization. dND-PG-Gd (III) complex demonstrated superior T1 relaxivity over an extensive number of magnetic fields and high dispersibility specifically in phosphate buffered saline making this complex a potential MRI contrast agent in

certain *in vivo* applications. Another study that aimed at investigating the polyvinylpyrrolidone (PVP) protective shell coatings on Gd-DND particles reported PVP-Gd-DND suspension as a self-assembling system linked by non-covalent bonds. This complex allows DND's to disperse without surface destruction and limited particle clustering forming a protective coating thereby preventing the collapse of these complexes in solutions possessing high ionic strength. These suspensions exhibit higher relaxivities in 8 T magnetic field than the most compounds reported in studies and are expected to have even greater relaxivities from 1 to 3 T magnetic fields usually employed in clinical MRI scanners. PVP-Gd-DND saline suspensions are biocompatible and stable. This suspension complex takes longer to enhance signals in the tissue. This is especially advantageous for diagnosing pathological problems that have a requisite for specific factors including greater spatial resolution and powerful signal enhancement along with slower excretion compared to other commonly used contrast agents. Furthermore, the concentration of gadolinium needed for imaging is significantly reduced when conjugated with the PVP shell therefore suppressing any possible GD3+ toxicity improving biosafety drastically [232].

Over the past decade, two categories of paramagnetic metal ions grafted DND particles have been observed to be propitious candidates as MRI contrast agents for *in vivo* medical imaging applications. One strategy includes the conjugation of various organo-gadolinium moieties to NDs and a conjugate system consisting of DND-polyglycerol-Gd (III) [233]. The increase in the interaction between water and the unpaired electrons of the Gd (III) ion facilitated by many coordination sites causes decline in the time of spin-spin relaxation and proton spin-lattice [234]. Although the vast majority of MRI contrast agents developed and used are Gd-based complexes, due to the potent toxicity of unbound Gd3+ ions, alternative contrast agents like manganese conjugated complexes are explored for MRI imaging. Similarly, the other category involves transition metal cations (Cu, Co, Gd, Mn) directly grafted to the surface of DND particles replacing the protons of carboxyl groups on the surface. Mn2+ demonstrates a relatively high magnetic moment (∼6μB) and also contributes to an efficient contrast enhancement and greater relaxivity [235]. Additionally, in contrast to gadolinium, it is a naturally available constituent and an enzyme or receptor cofactor. In spite of some risks that over accumulation of Mn^{2+} ions could pose, they are still considered promising as there exists a possibility of employing nanocarriers to retain these ions until release to target specific targets. In this regard, Panich *et al* recently examined the properties of Mn-grafted DND particles by measuring factors consisting of relaxivities, rates of proton relaxation along with the MRI images incorporated in 16 aqueous and saline suspensions of these NDs mimicking the biological environment of the human body [236]. The relaxation rates were found to be linearly related to Mn ion concentration. In the examined compounds, relatively quick relaxation rates and high relaxivities were obtained. Interestingly, when the aforesaid parameters in Mn-grafted DNDs are compared to those in recently studied Gd-grafted DNDs and Dotarem, a regularly used contrast agent in clinical practice, it became apparent that Mn-grafted DNDs could be used as novel contrast agents for MRI.

7. Future perspectives

Future investigations are expected to focus on the refining of sophisticated surface engineering approaches to improve the biocompatibility of NDs. Developing biocompatible modifications to reduce possible cytotoxicity would improve NDs' general appropriateness for therapeutic uses, including drug delivery and imaging. It is anticipated that surface engineering for NDs will become more customized to meet certain biological needs. Investigating multifunctional ND platforms offers a potential path for combining therapeutic compounds, targeting ligands, and imaging agents on a single ND surface to create flexible platforms for theranostic uses. NDs' imaging performance can be enhanced through pioneering surface modifications aiming at improved specificity, contrast, and stability in imaging modalities such as MRI, photoacoustic imaging, and fluorescence. The creation of ND surfaces to induce reactions to certain stimuli or environmental signals is suited for a prospective study. Dynamic biomedical applications might greatly benefit from responsive NDs because they may release therapeutic molecules in response to physiological changes or experience changes in optical or magnetic characteristics.

In vivo tracking and monitoring applications are expected to benefit from advances in ND surface engineering. This can include creating surface modifications that make it easier to track biological processes, cellular absorption, and dispersion in living things in real-time. It is also being considered to design ND surfaces for increased biodegradability. One potential approach to address concerns over long-term accumulation within the body is to formulate surface changes that facilitate the slow dissolution of NDs over time.

Future advancements might include the blending of NDs with other nanomaterials to create hybrid structures with complementary qualities. Incorporation of NDs with naturally occurring bioactive components such as phytochemicals can also be used as a chemoprotective agent against cancer [237]. These flavonoids are extracted from naturally occurring honey products like royal jelly and propolis [238]. The amalgamation of the distinctive

attributes of NDs with those of other nanomaterials holds promise for pioneering solutions across various biomedical applications. We must emphasize that the area of ND research is dynamic, with ongoing developments driven by a combination of technical innovation, multidisciplinary partnerships, and a deeper understanding of the material's intrinsic features. Scholars worldwide are now investigating new directions for utilizing NDs in medicinal settings, indicating that a combination of these variables will probably drive future advancements in ND surface engineering.

8. Conclusion

This study comprehensively discusses the usage of NDs in numerous biological applications, as well as their benefits and drawbacks. ND has been synthesized and characterized using a variety of approaches. The surface functionalization of ND with different moieties makes them effective for a wide range of biological applications. The unique features of NDs have piqued the curiosity of researchers in a variety of application domains. The importance of ND in diagnostics and medicine delivery systems has eclipsed its usage as a bright state-of-the-art. ND serves as a crucial nanovehicle for drug and gene delivery systems since it is naturally composed of carbon, which makes it less dangerous in the *in vitro* and *in vivo* environments. This review thoroughly examines the role of ND in anti-cancer treatment. It also covers how ND has been linked to several cellular uptake mechanisms for cancer treatment. ND has shown promising outcomes in the treatment of cancer stem cells as well as resistant cancer. It has also been widely used in the delivery of chemotherapy medicines. FND plays an important role in immunological activation and repression, which aid in cancer therapy. Fluorescent ND assists in the identification of tumors and the diagnosis of cancer. Its capacity to pass the blood-brain barrier and treat neurodegenerative illnesses has made it generally acknowledged. A new potential for biological usage has been examined because of the intrinsic structural integrity of NDs and the involvement of synthetic techniques like deaggregation and functionalization. However, cellular fate, limiting reaggregation, enhancing surface chemistry control, and generating NDs in vast quantities are all major concerns. On the bright side, there are a plethora of prospective applications that will propel scientific research forward.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contribution

The manuscript was written by T D A G, C J C, A S, S P, A B, and L N collected the information, drafted synthesis, mechanistic insight and figures. S K, M S, V R proofread and finalized the draft. K K and S R critically revised the manuscript, proofread and finalized it. All authors have read and approved the final version of the manuscript for submission to this journal.

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